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ORIGINAL ARTICLE

The use of surface alkaline phosphatase activity in the seagrass *Posidonia oceanica* as a biomarker of eutrophication

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Abstract

Eutrophication is one of the most relevant man-induced changes occurring in coastal waters. The identification and assessment of specific responses to eutrophication in seagrasses can provide a useful tool for the detection of changes in the water quality in coastal zones, given the wide range of distribution of these organisms. In this study, we combine a correlational (across-sites comparison) and a manipulative (fertilization experiment) approach to evaluate the usefulness and potential of alkaline phosphatase activity (APA) in the endemic Mediterranean seagrass *Posidonia oceanica* as an eutrophication biomarker. Our results showed that APA decreases promptly following nutrient additions, the response being maintained except during the winter period. APA also varies across natural meadows under different levels of nutrient discharges at scales relevant for monitoring purposes. AP activity seems to be an optimal 'physiological biomarker' that responds promptly and reliably to a pulse of eutrophication exposure. However, other considerations, such as the seasonality (the response disappears in winter), suggest its use with some caution and, as far as possible, as a complement of other bio-indicators.

Problem

Eutrophication is recognized as one of the major problems affecting both freshwater and coastal marine environments. Excess nutrients, resulting from diverse causes (domestic wastes, industrial discharges, agriculture, aquaculture) alter the ecosystem function and integrity, and represent a serious threat to aquatic environmental quality. The development of early warning indicators of eutrophication would be of great help to managers to take action before ecosystem degradation occurs. Biomarkers (defined as the quantitative and sublethal measures of changes in the biological system that respond to exposure to substances potentially causing undesired biological effects) can be useful in this respect, specially considering that the term 'biomarker' refers to the cellular, biochemical, molecular or physiological responses which precede those occurring at organismic or higher levels (Lam & Gray 2003). For a proper use of the biomarkers, especially for environmental monitoring purposes, information about the spatial and temporal variability of the biological measures used is vital. Biomarker responses can be significantly affected by natural seasonal fluctuations (*e.g.* Vizzini *et al.* 2003) or by excessive spatial variability within a given site (*e.g.* Morrisey *et al.* 1994) confounding the interpretation of the results if the sampling design is not adequate.

Seagrasses are among the most widely distributed organisms in coastal waters (Spalding *et al.* 2003). The growth and production of seagrasses are often limited by nutrients, both in tropical and temperate systems (Short *et al.* 1990; Pérez *et al.* 1991; Fourqurean & Zieman

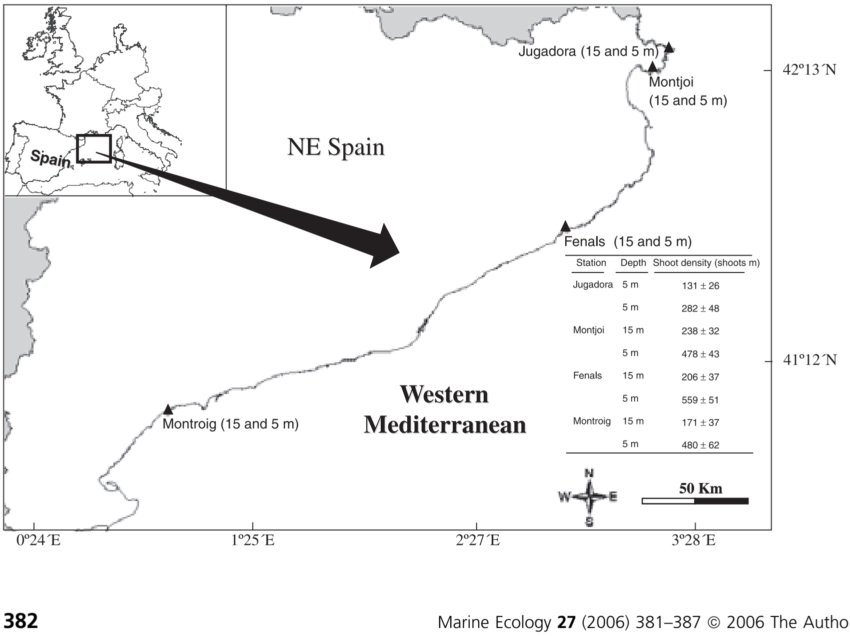
1992). Consequently, their physiology is especially sensitive to changes in nutrient availability (see review in Romero *et al.* 2006). Here we examine the potential as an eutrophication biomarker of the nutrient-sensitive enzyme Alkaline Phosphatase.

Alkaline phosphatase is a ubiquitous enzyme, mainly located in the external membrane or periplasmic space of the primary producers, able to hydrolyse organic phosphorous compounds (monoester phosphates). The released phosphate can be further taken up to meet the plant's nutritional demands. Alkaline phosphatase activity (APA) increases under phosphorous limitation in phytoplankton (Gage & Gorham 1985; Steinhart *et al.* 2002), macroalgae (Hernández *et al.* 1992; Lapointe 1995; Schaffelke 2001) and seagrasses (Pérez & Romero 1993; Invers *et al.* 1995). It has also been shown that APA in seagrasses decreases in the presence of increased phosphate concentration, both experimentally and along natural gradients (Pérez & Romero 1993; Invers *et al.* 1995). This supports the contention that APA is a potential biomarker of eutrophication. However, and before its use, additional knowledge should be obtained. To this end, we have combined a correlational and a manipulative approach to evaluate the usefulness and potential of APA in the endemic Mediterranean seagrass *Posidonia oceanica* as a biomarker of eutrophication, assessing: (i) the spatial variability within a given site and between locations suffering different nutrient discharges; (ii) the time-course of the response of APA to a nutrient enrichment (from days to 1 year), *i.e.* the time between the onset of the enrichment and the detection of the effects, and the long-term response of the plant to nutrient addition (ca. 1 year); and (iii) a possible seasonal behaviour of the response.

Material and Methods

Variability among meadows: correlational approach

A study of spatial variability in APA within the meadows and across the meadows affected and unaffected by nutrient discharges was conducted between December 2002 and January 2003 in four *Posidonia oceanica* meadows (Jugadora, Montjoi, Fenals and Montroig) in the NE coast of Spain (Fig. 1). In two of the meadows (Jugadora and Montjoi) no significant anthropogenic pressures were identified, while the other two (Fenals and Montroig) were affected by diffuse forms of urban and/or agricultural pollution (Agència Catalana de l'Aigua 2005). Each meadow was sampled at one shallow (ca. 5 m depth) and one deep (ca. 15 m) station. At each station, plants for APA determination were collected; other variables of interest, related to nutrient status, were also obtained from plant samples (see below), directly measured *in situ* (shoot density and meadow cover) or in sediment (organic matter and carbonate content) and pore water (nutrient concentration) samples. At each station, three different zones of 10 m^2, 25 m apart from each other, were randomly chosen along the same isobath. In each zone, four 0.16 m^2 (40 × 40 cm) quadrats were randomly placed to measure shoot density; nine 0.25 m^2 (50 × 50 cm) quadrats were placed 3 m apart from each other in three different transects randomly chosen to measure meadow cover, and 14 shoots of *P. oceanica* were collected, three superficial sediment vials, and three syringes containing ca. 25 ml of pore water were collected by SCUBA diving and transported in refrigerated seawater to the laboratory. Pore water samples were obtained using syringes with perforated cannula introduced 10 cm in the sediment.



Station	Depth	Shoot density (shoots m)
Jugadora	5 m	131 ± 26
	5 m	282 ± 48
Montjoi	15 m	238 ± 32
	5 m	478 ± 43
Fenals	15 m	206 ± 37
	5 m	559 ± 51
Montroig	15 m	171 ± 37
	5 m	480 ± 62

Fig. 1. Map of the situation of the meadows studied in the extensive survey. Some basic meadow features (shoot density at different sampling depths) are also included.

Experimental nutrient additions

The response of APA to nutrient increase was evaluated at one site (Fenals, see Fig. 1) in a shallow, patchy meadow (ca. 8 m depth) through a nutrient (N + P) enrichment experiment. Three treated (enriched) and three control plots were marked. In each enriched plot, nine nutrient-diffusing vials were placed (Fig. 2), six of them filled with sand and nitrate–phosphate–ammonium fertilizer, and the other three with OSMOCOTE, a slow-release fertilizer, ensuring thus short-term nutrient enrichment (days) as well as medium-term enrichment (1 month). The experiment lasted for 1 year (May 2003–June 2004), with a total of 11 nutrient addition events (ca. 1 per month). To better assess the time course of the response at early stages, five shoots were collected in each plot by SCUBA diving (at both the three treated and the three control plots) on days 4, 11, 22, and 29 after the first enrichment. Further, APA response was evaluated through ca. monthly samplings performed 1 day before each monthly nutrient enrichment.

Laboratory procedures: APA determination

In each zone, five shoots were collected (15 shoots per station). APA was determined in the second younger extended leaf of each sampled shoot. This leaf, commonly used for physiological analyses, is 25–50 days old (Alcoverro *et al.* 2001), ca. 20 cm length, and without conspicuous epiphytes that could confound the results (Pérez & Romero 1993). Each one of these leaves (0.2–0.5 g DW) was transferred into Pyrex bottles filled with 100–200 ml of assay medium composed by p-nitrophenyl phosphate, magnesium sulphate and seawater buffered with TRIS buffer at pH 9.7–10. After 2 h of incubation at 21–24 °C, under artificial saturating irradiance and constant agitation (80 rpm), the absorbance of the yellow hydrolysis product, p-nitrophenol, was spectrophotometrically measured at 410 nm (Pérez & Romero 1993; Invers *et al.* 1995; modified from Kuenzler & Perras 1965). Plants were then dried at 70 °C to a constant weight, and results were expressed as μmolPO_4^{-3} g DW^{-1} h^{-1}. It has to be reminded that the APA measured in this standard assay does not reflect *in situ* rates, but an estimate of the activity of the enzyme at saturating substrate and optimal pH conditions (Kuenzler & Perras 1965).

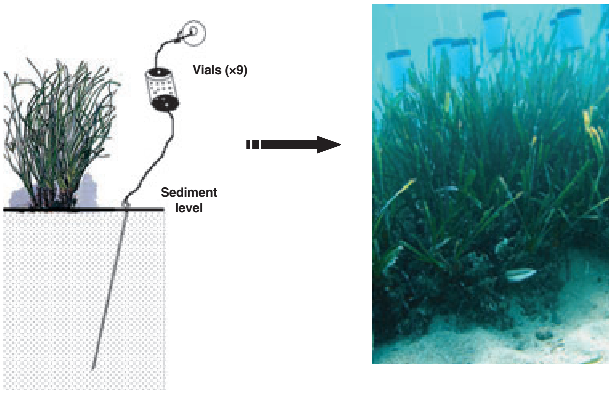


Fig. 2. Schematic illustration of the nutrient-diffusing vials placed in the enriched experimental plots. In each enriched plot, nine nutrient-diffusing vials were placed, six of them filled with sand and nitrate–phosphate–ammonium fertilizer, and the others three with OSMOCOTE, a slow-release fertilizer.

Other variables

In the extensive survey (correlational approach), other variables potentially helping to explain nutrient status were also measured in the shoots collected as described above.

All the leaves within one shoot of *P. oceanica* were measured to the nearest mm (length and width) in three shoots per zone (nine shoots per station); shoot size was expressed as cm^2 per shoot. Young leaves (the second younger extended leaf cleaned of epiphytes) and rhizomes were dried (70 °C to a constant weight) and finely ground. Carbon and nitrogen content were analysed in leaf tissues with a Carlo-Erba CNH elemental analyser, and P content in leaves and rhizomes was determined using inductively coupled plasma spectrometric analysis after acid HNO_3/H_2O_2 digestion (Cai *et al.* 2000). All results were expressed as % DW.

Organic matter in the sediment was measured as weight decrease after ashing (450 °C, 5 h) and carbonate content as CO_2 produced after sample treatment with HCl 10% using the Bernard calcimeter.

Pore water samples were extracted, filtered (Whatman GF/A) and kept frozen until analysed. Soluble reactive phosphorus, ammonium and nitrate concentrations were determined on an autoanalyser using standard methods (Grasshoff *et al.* 1983).

Data analysis

For data obtained in the extensive survey, differences between meadows and depths were tested with a three-way ANOVA, with meadow and depth being fixed factors and sampling zones being a random factor (nested in meadow). When a significant interaction was detected, a two-way ANOVA using only the fixed factors (meadow and depth) was carried out in order to allow *a posteriori* pair-wise comparisons of means using the Student–Newman–Keuls comparison test (SNK, Zar 1984). Prior to statistical analyses, the dependent variable (APA) was tested for normality and homogeneity of variances using the Kolmogorov–Smirnov test and Cochran test respectively. Despite the fact that data did not meet the condition of homocedasticity, the large sample size (n = 120) led us to consider ANOVA to be robust to departures from this assumption (Underwood 1997). Pearson correlations were performed between APA and different nutritional status descriptors and basic features of the meadow in order to evaluate their influence on the enzyme activity.

The assessment of APA response to water enrichment was carried out through Student's *t*-test analyses performed at each sampling time, comparing means between control and enriched plots.

Results

In the extensive survey, APA differed among meadows and among sampling depths (Table 1), but was homogeneous within a given meadow and depth (*i.e.* it did not vary among zones). APA was generally higher in shallow stations than in deep ones (Fig. 3). However, differences among shallow and deep stations varied from ca. twofold in Jugadora to non significant in Montjoi (SNK test). APA was in general higher in meadows without significant anthropogenic pressures (Jugadora and Montjoi deep station) and smaller in meadows affected by diffuse forms of urban and/or agricultural pollution (Fenals and Montroig). APA was significantly and negatively correlated with the phosphorous content of both rhizomes and leaves and positively with meadow cover, carbonate content in sediments, and ammonium concentration in the interstitial water of the sediment (Table 2).

The results of the fertilization experiment (Fig. 4) indicated that APA was significantly depressed by nutrient addition from the first measurement, that took place in May, 4 days after the treatment onset, and differences between treated and control plots subsisted until September (although with only marginal significance in two cases). These differences disappeared from October onwards (fall, winter and early spring), and APA attained minimum values in winter. At the end of the experiment there was a sudden switch in the sign of the response. This unexpected result could however be an artefact resulting from the repeated cropping, as the experimental plots were somewhat smaller than the control plots.

Table 1. Three-way ANOVA results (in bold significant values at $P < 0.05$) performed to test the importance of different potential source of variability on alkaline phosphatase activity in the correlational approach.

source of variability	df effect	MS effect	df error	MS error	F	P-value
meadow (M)	3	21.62	8	0.732	29.52	**0.000**
depth (D)	1	92.08	8	0.314	292.80	**0.000**
zone (Z)	8	0.73	96	1.137	0.64	0.739
interaction M × D	3	23.54	8	0.314	74.85	**0.000**
interaction D × Z	8	0.31	96	1.137	0.28	0.972

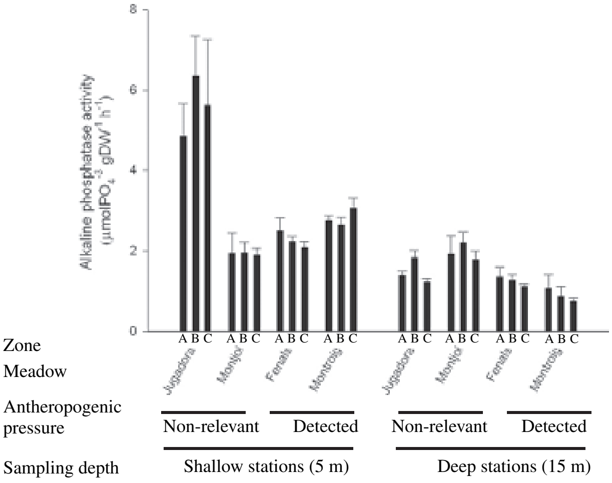


Fig. 3. Mean ± standard error of alkaline phosphatase activity values measured in the correlational approach. A, B and C correspond with the three different zones sampled in each sampling site. Anthropogenic pressure (non-detected and relevant), sampling depth and meadow are also included.

Table 2. Coefficient of linear correlation between alkaline phosphatase activity (APA) and descriptors related to nutritional status of the meadows and between APA and basic meadow features that could influence APA spatial variability (in bold significant values at $P < 0.05$) in the extensive survey (n = 24). As a result of sampling complications data of pore-water nutrients not measured in one site (Montjoi), correlations between them and AP activity are presented without considering this sampling site (n = 18).

variable	Pearson r	P-value	n
N leaves (% DW)	−0.18	0.398	24
P leaves (% DW)	**−0.40**	0.050	24
P rhizomes (% DW)	**−0.58**	0.003	24
leaf C/P	0.34	0.107	24
leaf C/N	0.05	0.801	24
leaf N/P	0.13	0.550	24
shoot size (cm^2/shoot)	0.21	0.334	24
density (shoots m^{-2})	0.22	0.869	24
cover (%)	**0.64**	0.036	24
OM in sediments (% DW)	0.04	0.307	24
carbonate sediments (% DW)	**0.43**	0.001	24
phosphate in the interstitial water of the sediment (μmol l^{-1})	0.33	0.179	18
ammonium in the interstitial water of the sediment (μmol l^{-1})	**0.62**	0.006	18
nitrate in the interstitial water of the sediment (μmol l^{-1})	0.41	0.092	18

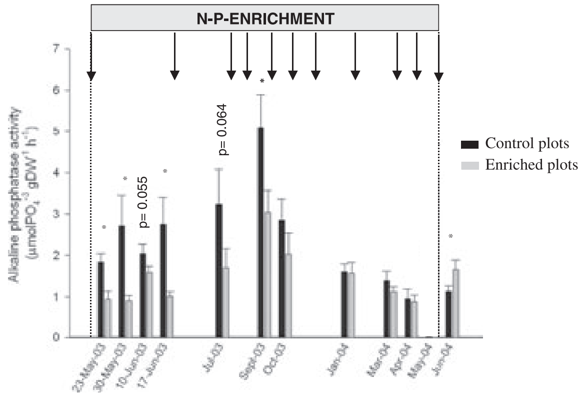


Fig. 4. Temporal variability of alkaline phosphatase activity response. Asterisks indicate significant differences ($P < 0.05$) between control plots and enriched plots (Student's *t*-test).

Discussion

Alkaline phosphatase activity in *Posidonia oceanica* shows some useful properties as eutrophication biomarker. First, it varies between meadows or part of meadows of different characteristics (*e.g.* different nutrient availability, different depth) but not within zones of the same meadow and depth. This fact is essential for the design of monitoring programmes, such as those foreseen during the implementation of the Water Framework Directive. Second, it responds consistently and predictably to nutrient availability in natural conditions (*i.e.* phosphorous nutrient content of plants correlated with APA across sampling stations) and under experimental treatments (AP activity decreased on plants from fertilized plots most of the year). The absence of correlation with soluble reactive phosphorous concentration in pore water highlights the importance of these integrative measures (*i.e.* APA or tissue nutrient content) compared to discrete observations to adequately reflect the environmental nutrient availability. Third, AP activity seems to be highly sensitive, with a fast response time (a few days).

Alkaline phosphatase activity increases in phosphate-deficient medium and decreases at high phosphate availability. When phosphate concentration is high, its uptake causes a high tissue phosphorous content and, consequently, APA shows a strong negative response to internal phosphorous concentration. Moreover, high external phosphate concentration does not totally suppress APA, suggesting the existence of two different enzyme pools: one adaptive, regulated by phosphate concentration, and the other constitutive, independent of external phosphate levels that might interact (Hernández *et al.* 2002). However, total AP activity shows an unquestionable response to phosphate availability, confirming its adequacy as a biomarker of eutrophication.

Nevertheless, there are three additional sources of variation that should be considered before using APA as a eutrophication biomarker. The first one is seasonality. In effect, APA not only varies seasonally following nutrient availability (see values of the control plants in Fig. 4) but also, at least in the conditions of our experiment, the response induced by the nutrient increase disappears in winter. At this time of the year, leaf growth in *P. oceanica* is reduced and the nutrient availability relatively high; hence, nutrient demand is at its minimum. The seasonal APA response thus reflects the existence of seasonality in the extent of nutrient limitation in *P. oceanica* (Alcoverro *et al.* 1997). However, in the correlational approach, performed during winter, APA was able to reflect differences in the nutrient availability among meadows and/or depths. Possibly, APA measured in winter reflects the activity of the constitutive pool, which depends on meadow features such as the cover (higher nutrient demands in meadows which support a higher shoot and belowground tissue production) or the carbonate content in the sediment (P absorption to calcium carbonate minerals, see below); these contentions are supported by the correlations found (Table 2).

A second source of variation that should be taken into consideration is depth. Shallow meadows do not only inhabit in an environment with lower nutrient availability (shallow waters characteristically have lower nutrient concentrations: Romero *et al.* 1998), but they also have higher nutrient requirements because of higher shoot densities and meadow cover (Alcoverro *et al.* 1995). Moreover, leaf biomass losses are higher in shallow meadows because of higher herbivore pressure (Tomás 2004) and higher hydrodynamics (Krause-Jensen *et al.* 2000; Middelboe *et al.* 2003), resulting in additional nutrient deprivation. Consequently, important differences in nutrient imbalances exist between shallow and deep meadows, a fact which is well reflected in APA values.

Finally, a third source of variation is the sediment carbonate content. P-limitation in seagrasses is generally associated with environments with carbonate sediments because of the P absorption by calcium carbonate minerals (Short *et al.* 1990; Fourqurean *et al.* 1992; Chambers *et al.* 2001; but see Erftemeijer & Middelburg 1993). In this study, the significant correlation between AP activity and sediment carbonate content suggests that the effects of chronic P pollution could be partially attenuated by these carbonates, resulting in high APA values despite receiving direct nutrient inputs.

In conclusion, the measurement of APA presents advantages that can help in the diagnosis of eutrophication (*e.g.* high sensitivity, low spatial variability at small scales and consistency in the response). However, because of the shortcomings such as the seasonality in the response, caution should be exercised when using AP activity as a biomarker. Additionally, the very fast response of APA (with maximum response being attained in only a few days) impedes the discrimination between sporadic nutrient pulses and a sustained increase in nutrients. The combination of APA with some other bioindicators such as nutrient content and isotopic signatures (*e.g.* Udy & Dennison 1997) can improve the usefulness of APA as a biomarker of eutrophication.

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