Magnitude of herbivory in *Posidonia oceanica* (L.) Delile and factors responsible for spatial variation

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The effects of nutrient enrichment on oligotrophic seagrass meadows: an integrative experimental approach

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RESUM:

Els nutrients juguen un paper fonamental en els ecosistemes de fanerògames marines, controlant i modificant processos fisiològics, modificant factors a nivell individual i poblacional i potencialment impactant altres ecosistemes. Per tant, una aproximació integrativa és essencial per comprendre el funcionament de' ecosistema.

En aquest estudi es presenta un experiment de fertilització de la columna d'aigua conduït a una praderia oligotròfica exposada de *Posidonia oceanica* (L.) Delile (Mediterrània noroccidental) on es va mesurar l'estacionalitat dels diferent components (biomassa de la planta i dels epífits, blooms de macroalgues) i dels processos (limitació de nutrients, pressió de l'herbivorisme i ombrejat de les fulles) que són susceptibles de ser modificats directa o indirectament pels nutrients. A més, per una millor comprensió de com els nutrients poden controlar possibles canvis en la biomassa de la planta es va conduir un seguiment estacional del contingut de nutrients a la planta i als epífits. Els nutrients van estimular el creixement i la biomassa foliar durant tot el període de limitació de nutrients (primavera) i van incrementar l'herbivorisme del peix *Sarpa salpa* (L.) durant l'estiu amb la subsegüent reducció de biomassa foliar i reserves de carbohidrats. Altres processos sovint esmentats com l'ombrejat de les fulles pels epífits o els blooms de macroalgues van tenir poca rellevància.

Malgrat aquesta complexa xarxa d'interaccions, el subministrament de nutrients no va produir modificacions importants en la biomassa de la planta i en les reserves de carbohidrats a un termini anual. Els ecosistemes *Posidonia oceanica* a zones oligotròfiques i condicions de corrents moderades – com les dominants en la regió d'estudi –, són considerablement resistents a l'eutrofització.

INTRODUCTION

The ever-increasing eutrophication in coastal areas is causing significant alterations in marine near-shore ecosystems. Some of them are rather conspicuous, but subtle changes in ecosystem functioning are probably more common, although not less important. This is particularly true for systems that are characterized by naturally low nutrient concentrations, including freshwaters (Hillebrand 2002, Worm et al. 2002), coral reefs (Thacker et al. 2001, McCook 2001, Szmant 2002), benthic macroalgae (Belegratis 1999), and seagrass beds (McGlathery 1995, Delgado et al. 1999). Nutrients play a vital role in ecosystems by controlling and modifying physiological aspects, affecting individual and population-level factors, and potentially impacting ecosystem processes (see reviews in Smith et al 1999, Romero et al. 2006). In oligotrophic seagrass ecosystems a fair understanding of how nutrients affect the most relevant of these individual aspects has been achieved (Romero et al. 2006). However, much remains to be investigated on how these multi-level processes are interacting and responding in a more integrative scenario (but see Ferdie & Fourqurean 2004).

Nutrients can modify the properties and behaviour of primary producers and consumers in seagrass systems through a sequence of flow-on effects at different levels that go from the physiological through the individual to the ecosystem (see Fig. 1 for a conceptual model). The effects of nutrient availability on seagrass physiology (e.g. nutrient uptake, nutrient content and storage), growth and biomass have been widely investigated (see review in Romero et al. 2006). Changes in the availability of nutrients may induce an adjustments in the photosynthetic capacity (Alcoverro et al. 2001), in the nutrient quota (Moore & Wetzel 2000) or in the levels of carbohydrate content (Invers et al. 2004), which in turn cause modifications in growth rates and/ or in structural meadow features (Fitzpatrick & Kirkman 1995, Moore & Wetzel 2000, Peralta et al. 2002). Nutrients can also influence primary producers other than the seagrass itself , inducing the development of fast-growing algae (Valiela et al. 1997) or the epiphyte overgrowth (Neckles et al. 1993, Coleman & Burkholder

1995, Dalla Via et al. 1998). An increase in epiphyte and/ or macroalgae biomass often results on a reduction of the light reaching the seagrass canopy due to shading (Dalla Via et al. 1998, Brun et al. 2003; but see Alcoverro et al. 2004). Changes in palatability as a result of nutrient enrichment in both seagrass and epiphyte communities have also been shown to alter herbivore preference and behaviour. They are the consequence of modifications in the nutritional value of epiphytes or seagrass leaves (Duarte 1990, Shepherd 1987, McGlathery 1995, Delgado et al. 1999), of changes in epiphytic composition (Gacia et al. 1999) or of changes in chemical defences (Lubchenco & Gaines 1981). In turn, an increase in herbivory appears to either mitigate or enhance deterioration of seagrass habitats, depending on whether consumption reduce epiphyte and macroalgae shading or the seagrass plant biomass (Cambridge et al. 1986, Ruiz et al. 2001, Williams & Ruckelshaus 1993, Hays 2005).

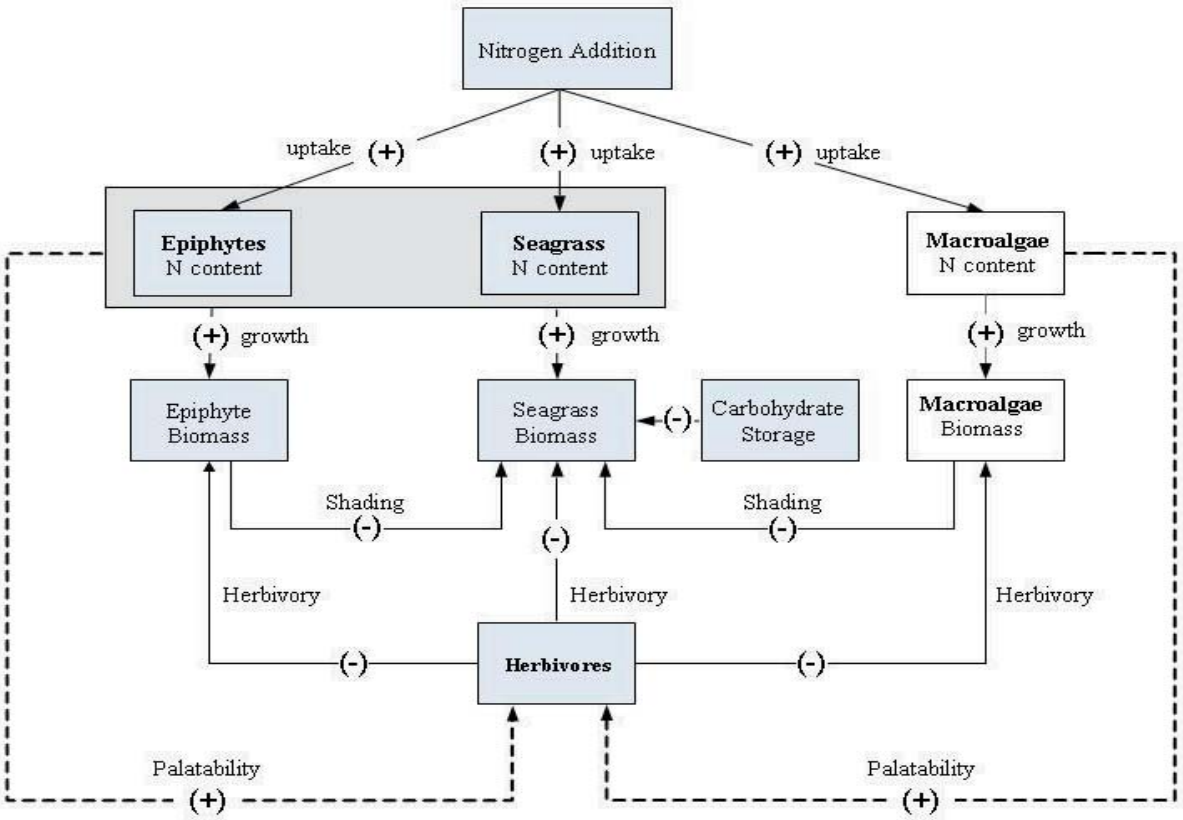

Fig. 1. Initial working hypothesis including all possible flow-on effects in *Posidonia oceanica* meadows following nutrient increase.

The dominant Mediterranean seagrass *Posidonia oceanica* (L.) Delile seems well adapted to nutrient-poor waters, and may be severely affected by coastal eutrophication. For this plant, nutrient limitation occurs in late spring, when nutrients are scarce and leaf elongation is high, while in winter nutrient luxury consumption and storage takes place (Alcoverro et al. 1997). Epiphyte load is maximal in spring-summer, depending on the depth (Alcoverro et al. 1995), and leaf consumption by the main macroherbivore, the fish *Sarpa salpa* (L.) is particularly high in summer (up to 50% of the biomass in some areas) in shallow meadows (Prado et al. in press). This marked seasonality in the behaviour of most ecosystem components adds a further level of complexity to an already complex set of interactions, which needs clearly to be considered at least within an annual perspective.

In this study, we used an experimental approach, consisting in monthly field nutrient enrichment over one year, to test some key aspects of the interaction model summarized in Fig. 1. The experiment was performed on a shallow *Posidonia oceanica* bed, with the aim of determining which processes, and within which season, were the most sensitive to nutrient availability changes in such oligotrophic meadows. We examined the direct influence of nutrient addition on seagrass, epiphytes and macroalgae at different levels, and we investigated potential indirect impacts of nutrient enrichment such as epiphyte or macroalgal shading and herbivory.

MATERIALS AND METHODS

Study site and experimental design. The study was conducted from May 2003 to June 2004 on an 8-meter deep *Posidonia oceanica* meadow off Fenals beach, in the northeast Mediterranean Spanish coast (see Fig. 2). The beach is located in an open rocky shore with low current regime and generally light wave conditions, except during storm episodes. Water column levels of nutrients in the area are relatively low, with annual averages of 0.96 ± 0.07 μM for nitrate and 0.29 ± 0.04 μM for phosphate (Cebrián et al. 1996).

We divided the study area into two zones, which differed in the meadow structural features. In the first one (zone A in Fig. 2), the seagrass appeared with large and coalescent patches of *Posidonia oceanica*, while in the other (zone B in Fig. 2) the seagrass appeared in smaller (1-4 m^2 in surface), isolated patches. In zone B, 6 patches with a surface area of 2-3 m^2 were selected, three of them were randomly chosen and treated with nutrients, and the other three maintained as controls. In zone A, we selected and marked six areas of 2-3 m^2 surface (as in zone B) within six larger different patches, and treated as described above. We maintained a distance of at least 10 m between experimental plots to avoid contamination of controls by nutrient additions. As the spatial distribution of the herbivorous sea urchin *Paracentrotus lividus* (Lmk.) was highly clumped in the study area, and to reduce as far as possible this source of variability, we manipulated the number and size frequency distribution of sea urchins to reach maximum possible homogeneity among plots. Density was then driven to 9-12 individuals per m^2 in each plot, which is the average density in the area, and surveys were periodically performed to ensure that the number and size frequency distribution of sea urchins was maintained throughout the experimental period.

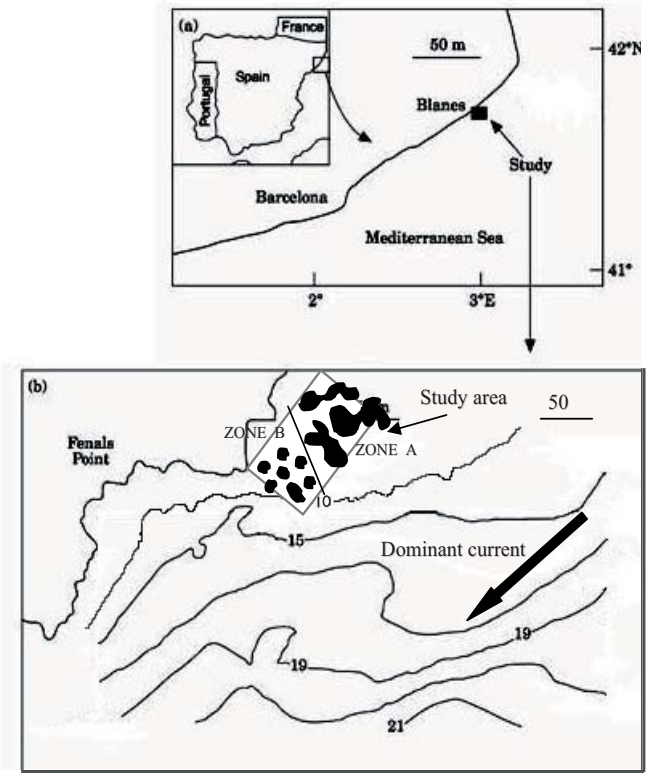

Fig. 2. Map of the northeast Mediterranean Spanish coast showing the position of the study area at Fenals point and the two seagrass areas with distinctive morphological features.

Nutrient addition experiment. After preliminary laboratory tests, we used as fertilizer both a mixture of di-Potassium Hydrogen Phosphate anhydrous, Sodium nitrate and Ammonium chloride (Panreac) and Omocote universal fertilizer (ratio N-P-K: 14-13-13). Nutrients were mixed with sand (50% each) and included in plastic containers (200 ml) whereas the Osmocote fertilizer was simple topped up within containers. The cylindrical external face of the containers was holed punched up to 20 times (2 mm hole diameter) to ensure a slow nutrient release. This method was able to release nutrients during 1 week (the salts mixture, personal observations) and 1 month (the Osmocote, Heck et al. 2000), respectively. At each enrichment event, a combination of 6 nutrient and 3 Osmocote containers were deployed per plot after sample collection. The combination of both methods (nutrient containers and Osmocote) allowed an adequate nutrient supply in the field throughout the year (from May 2003 to May 2004) with a single monthly addition. No decreased water clarity in the form of either particulate matter or phytoplankton blooms was observed at any time as a result of the nutrient manipulation.

Data collection and samples processing. Data were obtained following the conceptual model in Fig. 1. Seagrass shoot size and epiphyte biomass, carbon and nitrogen content in seagrass leaves and epiphytes, and carbohydrate content in seagrass rhizomes were evaluated monthly. To this end, six replicate shoots were randomly collected in monthly sampling campaigns at each one of the 12 plots. Shoots were sorted into leaves and rhizomes. Seagrass shoot size was assessed by sorting the leaves and measuring their length and width. Epiphytes were removed from leaves using a razor blade (Dauby & Poulicek 1995) and leaves and epiphyte samples were separately dried at 60ºC during 48 h, weighted and grounded to fine powder to determine their C and N content with an EA 1108 CHNS-O Carlo Erba Analyser. The first centimetre of rhizome was cut off, cleaned from the old scales, and then dried at 60ºC during 48 h and grounded to fine powder. In the dry ground samples soluble carbohydrates and starch were extracted in hot (80ºC) ethanol and then analysed as in Alcoverro et al. (1999).

Macroalgal cover was also monitored monthly by visuals surveys, within the plots

and in the surrounding zones.

Leaf growth was determined at the beginning of the experiment (June 2003), in March 2004 (when plants are nutrient-sufficient) and in June 2004 (when nutrient limitation occurs) with the leaf marking method described in Romero (1989). In each plot, 5 shoots were selected at random, marked with a needle and collected one month later. Shoot growth is expressed in terms of leaf elongation (cm^2 $shoot^{-1}$ d^{-1}).

Leaf shading caused by epiphytes was determined in August (maximum epiphytic loading) by combining the general carbon balance model from Alcoverro et al. (2001) with the relationship between epiphyte biomass and light extinction from Alcoverro et al. (2004). Within-shoot distribution of epiphyte biomass was assessed in 7 shoots per treatment and the value of light extinction caused by epiphytes was obtained for each age-class and leaf side. We fed the carbon balance model with those data, estimating thus the reduction in carbon gains caused by epiphyte light shading in each plot.

Grazing pressure was assessed by estimating the frequency of bite marks left by the fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus* (Boudouresque & Meinesz 1982, Alcoverro et al. 1997).

Data analyses. Differences among treatments, zones, and among sampling periods were assessed using a mixed four-way ANOVA. Dependent variables were shoot size (in cm of total leaves' length), epiphyte biomass, %N in *Posidonia oceanica* leaves and epiphytes, number of bite marks (*Sarpa salpa* and *Paracentrotus lividus*) and number of intact leaves, leaf growth and content of total non-structural carbohydrate (TNC). Independent variables were treatment (fixed, fertilised vs. control), sampling time (fixed, month), zone (fixed, coalescent patches vs. small patches) and plot nested in zone (random).

Data were transformed when necessary to meet ANOVA assumptions of normality (Chi-square test) and homogeneity of variances (Cochran's test). For all analyses, the significance level was fixed at $p< 0.05$. SNK post hoc comparisons were used whenever necessary.

RESULTS

Overall, fertilisation decreased shoot size, with annual mean values of 69.2 ± 14.6 and 108.7 ± 22.4 cm^2 $shoot^{-1}$ in fertilised and unfertilised plots respectively. However, an important seasonality in the effects of the treatment was detected (i.e. significant Time x Month interaction), with values higher in the control than in the nutrient plots in July-August 2003 and the opposite (larger shoots in fertilised plots) in May-June 2004 (Table 1a, SNK test, $p < 0.05$). Seasonally, the lowest shoot size coincided with the period of maximum fish grazing (i.e. July-September; Tomas 2005a) and the largest with the period of higher aboveground production in May-June (Romero 1989; Fig. 3, Table 1a). A significant Month x Zone interaction was also observed as a result of slightly shorter shoots in Zone B (that formed by small, isolated seagrass patches) during May-June.

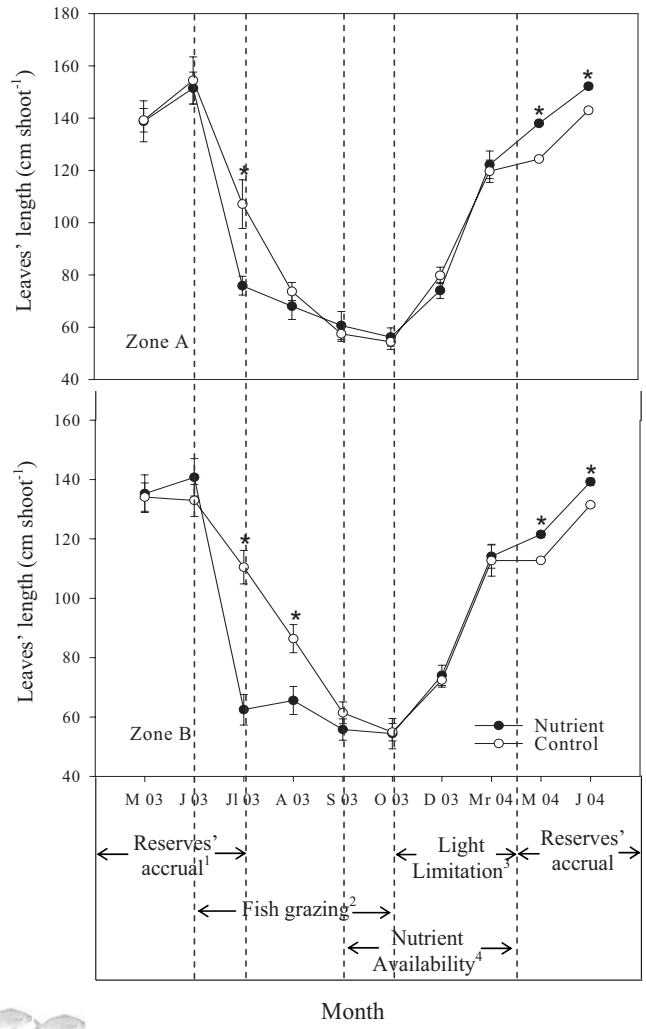

Fig. 3. Monthly values of shoot size (cm of leaves' length $shoot^{-1}$). When values from nutrient and control plots differ significantly (SNK test), this is indicated by an asterisk. [1]Period of reserves accumulation: Alcoverro et al. 2001, [2]Period of fish grazing: Prado et al. in press, [3]Period of light limitation: Alcoverro et al. 2001, [4]Period of nutrient availability: Alcoverro et al. 1997.

Table 1. Analysis of variance (ANOVA) on a) shoot size (i.e. leaves' length), b) epiphytic biomass per shoot, and c) epiphytic biomass on the different tissue age classes of shoot's leaves (significant results are given in **bold**).

ANOVA		**Seagrass**			**Epiphyte biomass**							
Source of		a) *Shoot size*			b) *per shoot*			**Source of**		c) *per tissue age*		
variation	**df**	**MS**	**F**	***p***	**MS**	**F**	***p***	**variation**	**df**	**MS**	**F**	***p***
Month = M	9	444462	71.43	**0.0000**	0.1888	896.16	**0.0000**	Treatment = T	1	0.0018	527.76	**0.0000**
Zone = Z	1	3166	7.18	0.0535	0.0000	0.02	0.8911	Age-class = A	3	0.0013	374.23	**0.0000**
Treatment = T	1	878	12.14	**0.0253**	0.0027	14.21	**0.0196**	Leaf side = S	1	0.002	48.93	**0.0000**
Plot = P (T)	4	72	0.36	0.8340	0.002	0.79	0.5334	T x A	3	0.0012	361.49	**0.0000**
M x Z	9	400	2.47	**0.0260**	0.0059	27.86	0.1141	T x S	1	0.002	48.93	**0.0000**
M x T	9	1859	29.79	**0.0000**	0.003	1.75	**0.0000**	A x S	3	0.002	48.93	**0.0000**
Z x T	1	207	0.48	0.5297	0.005	5.25	0.0837	T x A x S	3	0.002	48.93	**0.0000**
M x P (T)	36	62	0.31	1.0000	0.002	0.89	0.6521					
Z x P (T)	4	438	2.21	0.0688	0.001	0.37	0.8324					
T x P (T)	-	-	-	-	-	-	-					
M x Z x T	9	176	1.09	0.3954	0.002	1.47	0.1962					
M x Z x P (T)	36	162	0.81	0.7637	0.001	0.62	0.9554					
M x T x P (T)	-	-	-	-	-	-	-					
Z x T x P (T)	-	-	-	-	-	-	-					
M x Z x T x P (T)	-	-	-	-	-	-	-					
		Cochran's *C*: 0.109 (n.s) Transformation: -			Cochran's *C*: 0.087 (n.s) Transformation: √√x					Cochran's *C*: 0.289 (n.s) Transformation: √x		

Treatment enhanced epiphytic biomass, but only during late summer (August-September, 0.08 ± 0.01 and 0.02 ± 0.005 g DW shoot^{-1} d^{-1} in nutrient and control plots, respectively; Fig. 4, Table 1b, SNK test, $p< 0.05$). In contrast, macroalgal cover was very low at all sites during all the experimental time (data not shown).

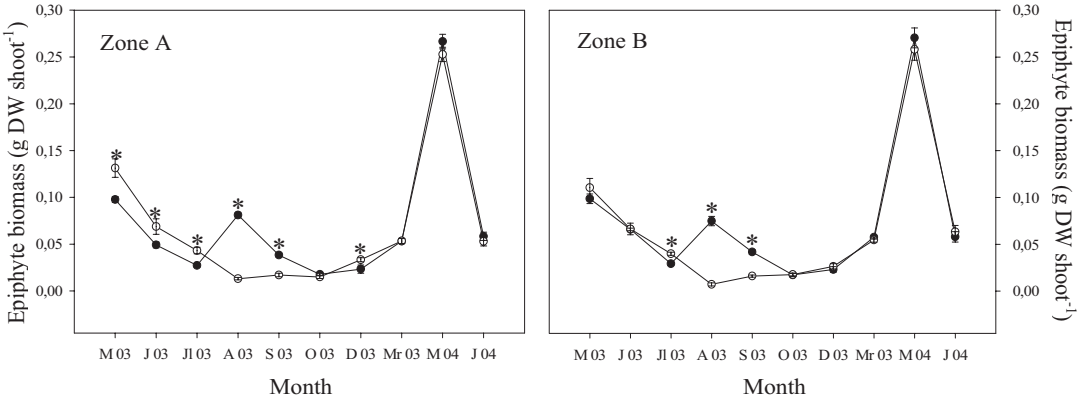

Fig. 4. Monthly values of epiphyte biomass (g DW shoot^{-1}) from nutrient and control plots at each study zone.

Nutrient enrichment caused an overall significant increase of the plant annual N content (1.9 ± 0.13 %N, and 1.6 ± 0.13 %N, fertilised and control plots, respectively). However, N content was clearly seasonal, with highest values in spring (May 2003, 2004) and lowest values at the end of summer (September 2003), as was the effect of the nutrient addition (i.e. significant Treatment x Month interaction), with an increase in the nutrient content in fertilised plots from June to December 2003 (Fig. 5, Table 2a, SNK test, $p< 0.05$) and then again in June 2004, but not the rest of the year. Spatial heterogeneity across the study site accounted for significant Month x Zone, Month x Plot and Month x Zone x Plot interactions but anyhow, spatial differences between Zones or Plots did not alter the direction of the Treatment effect. Nutrient enrichment also caused a slight decrease in the epiphyte nitrogen content during June-July 2003 followed by an important increase during August-September 2003 (significant Treatment x Month interaction; Fig. 5, Table 2b, SNK test, $p< 0.05$). Seasonal trends were similar to those of nitrogen in *Posidonia oceanica* with maxima in spring (May 2004, 2004) and minima in early fall (October 2003).

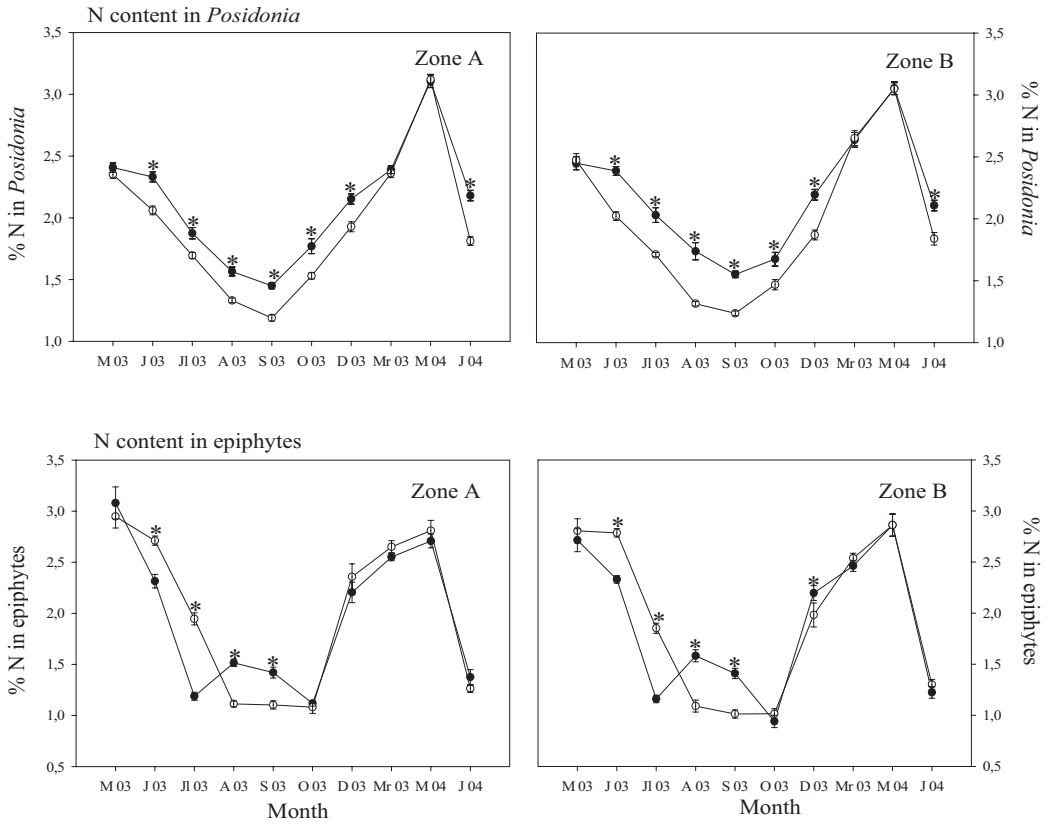

Fig. 5. Monthly values of N content (% DW) from both *Posidonia oceanica*, and epiphytes at each study zone. Significant differences (SNK) between treatment groups are indicated with asterisks.

Table 2. Analysis of variance (ANOVA) on a) N content in *Posidonia oceanica*, and b) N content in epiphytes (significant results are given in **bold**)

ANOVA		**a) N in *Posidonia oceanica***			**b) N in epiphytes**		
Source of variation	**df**	**MS**	**F**	***p***	**MS**	**F**	***p***
Month = M	9	9.9247	462.82	**0.0000**	18.843	242.4	**0.0000**
Zone = Z	1	0.1501	5.74	0.747	0.398	9.03	**0.0397**
Treatment = T	1	3.6663	191.67	**0.0002**	0.171	3.96	0.1173
Plot = P (T)	4	0.0191	1.40	0.2360	0.043	0.99	0.4138
M x Z	9	0.1737	8.10	**0.0000**	1.028	13.22	0.0720
M x T	9	0.0907	3.67	**0.0024**	0.105	1.97	**0.0000**
Z x T	1	0.0258	0.99	0.3764	0.004	0.10	0.7658
M x P (T)	36	0.214	1.56	**0.0269**	0.078	1.78	0.0061
Z x P (T)	4	0.0261	1.91	0.1098	0.044	1.01	0.4031
T x P (T)	-	-	-	-	-	-	-
M x Z x T	9	0.0211	0.85	0.5752	0.066	1.25	0.2957
M x Z x P (T)	36	0.0249	1.80	**0.0052**	0.053	1.21	0.1987
M x T x P (T)	-	-	-	-	-	-	-
Z x T x P (T)	-	-	-	-	-	-	-
M x Z x T x P (T)	-	-	-	-	-	-	-
		Cochran's *C*: 0.057 (n.s) Transformation: -			Cochran's *C*: 0.097 (n.s) Transformation: -		

Carbohydrate (sucrose and starch) content of rhizomes of *Posidonia oceanica* decreased in nutrient plots during summer (15 ± 0.9 and 18.7 ± 0.8% DW in nutrient and control plots, respectively from July to September; Fig. 6, Table 4b, SNK test $p < 0.05$), and the overall seasonal trend resulted in values ranging from 9.6 ± 0.9% DW in early spring to 17.7 ± 0.9% DW in early to mid summer.

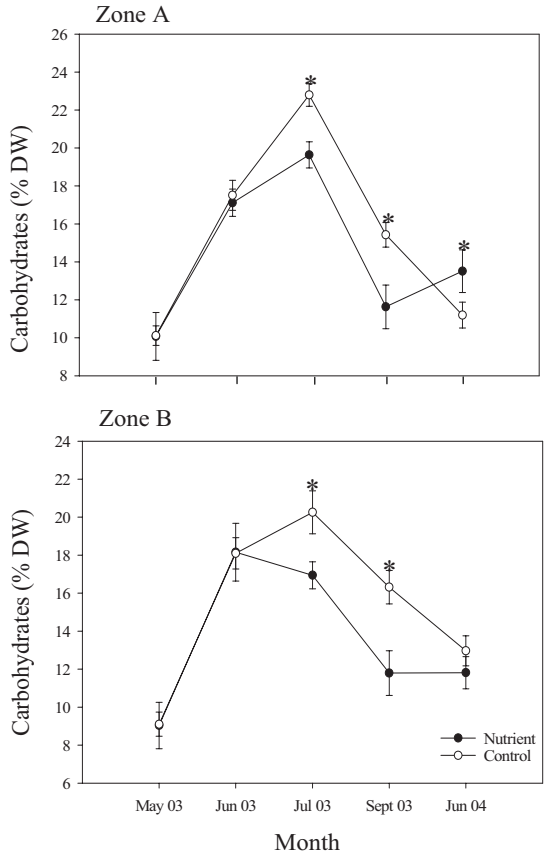

Fig. 6. TNC (Total non structural carbohydrates) in rhizomes of *Posidonia oceanica* in the two study zones obtained at the three sampling times. Significant differences between treatment groups (SNK) are indicated with asterisks.

The within-shoot distribution of epiphyte biomass was not affected by the nutrient treatment. Both in nutrient and in control plots, epiphyte biomass accumulated on the older tissues (i.e. tips of the older leaves), particularly on the external leaf sides, with values consistently higher in fertilised plots (Fig. 7a, Table 1c, SNK test, $p < 0.05$) than in control ones. Light absorption by epiphytes was generally low, except in the oldest age-class in nutrient plots, but this leaf class had little contribution to the net carbon gains (Fig. 7b). Therefore, the shoot carbon gains in treated plots were only 6.2% lower than in control plots.

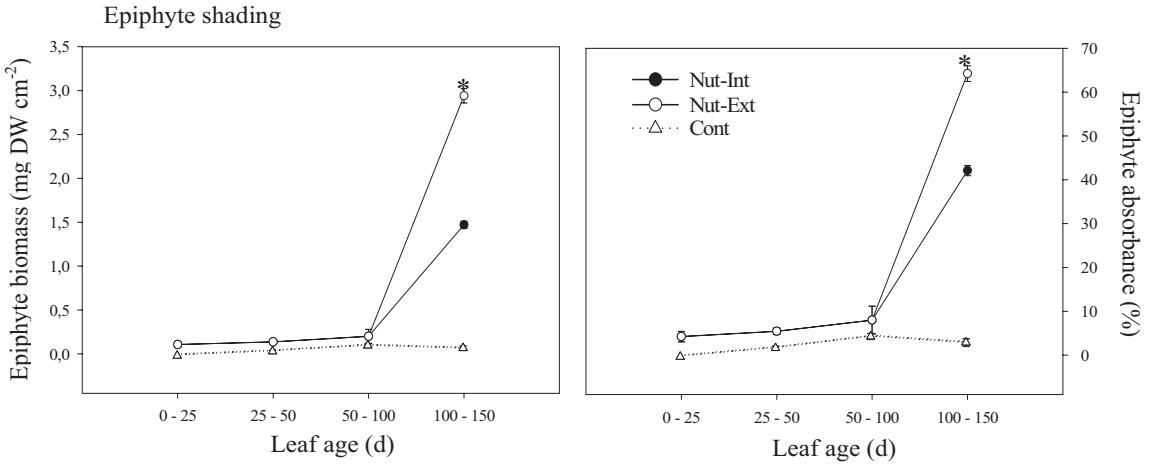

Fig. 7. Distribution of epiphytic biomass by tissue age on each leaf side (a), and light absorbed (400-700 nm) by epiphytes growing on each leaf tissue age and side of shoot's leaves in nutrient and control plots (b; data obtained from August samples). Significant differences between treatment groups (SNK) are indicated with asterisks.

Significant seasonal differences in the frequency of *Sarpa salpa* bite marks showed that the period of fish activity started in June, reaching its maximum from July to September and decreasing to a minimum in winter (Fig. 8, Table 3b, SNK test, $p< 0.05$). The number of *Paracentrotus lividus* bite marks also displayed significant seasonal differences (Fig. 8, Table 3b; SNK test, $p< 0.05$) but tended to be lower in summer, probably due to a masking effect by fish marks. For neither species, significant differences between treatments were observed. In contrast, the number of intact leaves was higher in control plots during summer (Fig. 8, significant Treatment x Month interaction in Table 3c, SNK test, $p< 0.05$).

Table 3. Analysis of variance (ANOVA) on a) number of *Sarpa salpa* bite marks per shoot, b) number of *Paracentrotus lividus* bite marks per shoot, and c) number of intact leaves per shoot (significant results are given in **bold**).

ANOVA		**a) *S. salpa* bite marks**			**b) *P. lividus* bite marks**			**c) Intact leaves**		
Source of variation	**df**	**MS**	**F**	***p***	**MS**	**F**	***p***	**MS**	**F**	***p***
Month = M	9	40.531	5.8497	**0.001**	11.549	11.117	**0.000**	16.443	23.073	**0.000**
Zone = Z	1	9.025	2.8525	0.1665	5.378	5.176	0.2380	0.278	0.270	0.6310
Treatment = T	1	11.736	1.6562	0.2675	2.500	2.406	0.1222	7.511	29.714	**0.055**
Plot = P (T)	4	7.086	1.1111	0.3519	0.689	0.663	0.6182	0.253	0.532	0.7122
M x Z	9	12.050	1.0667	0.4099	0.896	0.863	0.5593	0.568	0.733	0.6758
M x T	9	0.489	0.0706	0.9999	0.142	0.137	0.9986	1.406	1.973	**0.0721**
Z x T	1	4.669	1.4759	0.2912	1.111	1.070	0.3021	0.900	0.873	0.4029
M x P (T)	36	6.929	1.0864	0.3473	1.062	1.023	0.4401	0.713	1.500	**0.0406**
Z x P (T)	4	3.164	0.4961	0.7386	1.944	1.0872	0.1161	1.031	2.170	0.0731
T x P (T)	-	-	-	-	-	-	-			
M x Z x T	9	6.225	0.5511	0.8270	1.099	1.058	0.3951	0.2277	0.293	0.9721
M x Z x P (T)	36	11.297	1.7712	**0.0066**	1.003	0.966	0.5300	0.774	1.630	**0.0175**
M x T x P (T)	-	-	-	-	-	-	-	-	-	-
Z x T x P (T)	-	-	-	-	-	-	-	-	-	-
M x Z x T x P (T)	-	-	-	-	-	-	-	-	-	-
		Cochran's *C*: 0.085 (n.s) Transformation: -			Cochran's *C*: 0.050 (n.s) Transformation: -			Cochran's *C*: 0.076 (n.s) Transformation: -		

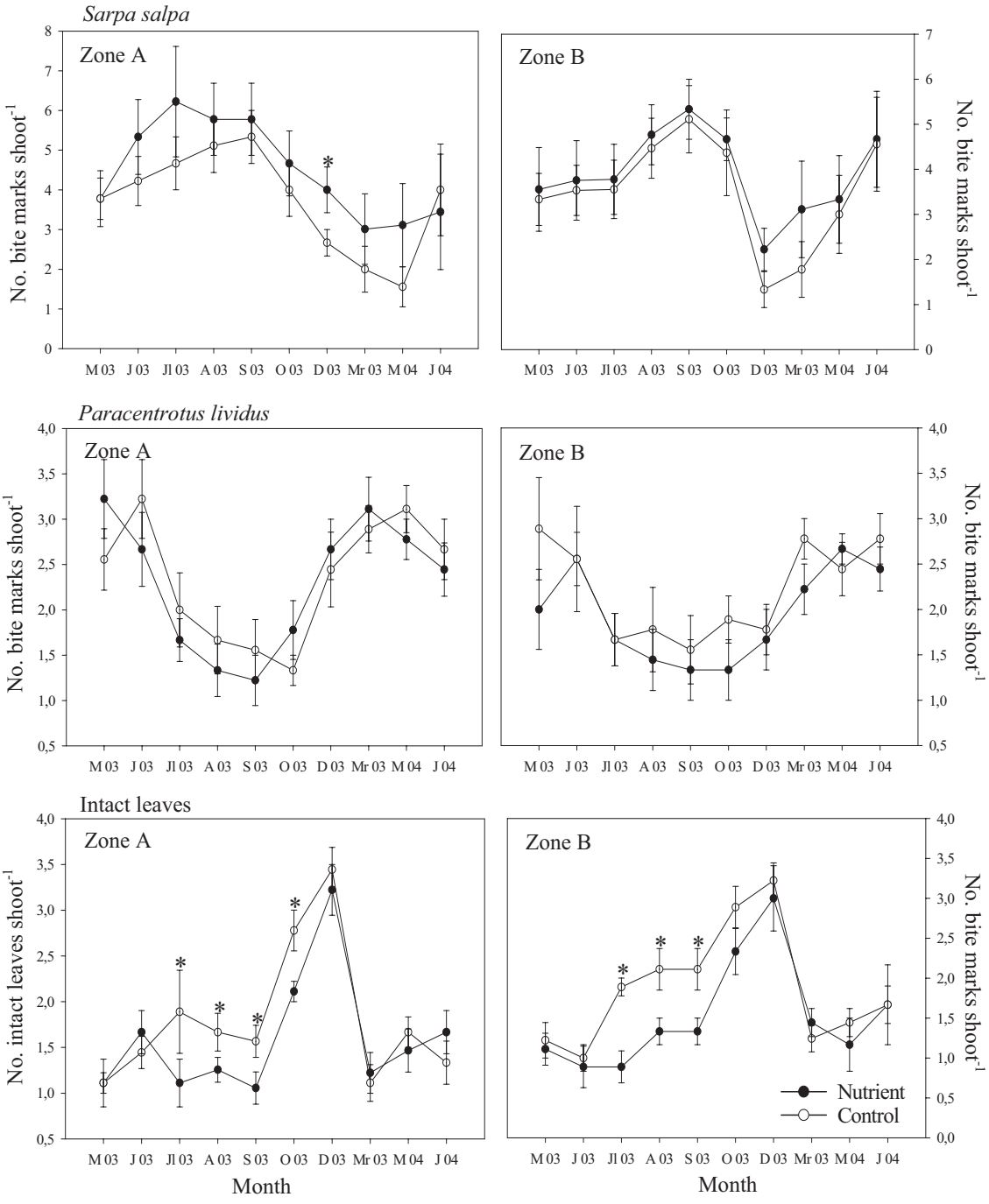

Fig. 8. Monthly rates of *Sarpa salpa* and *Paracentrotus lividus* bite marks per shoot, and number of intact *Posidonia oceanica* leaves per shoot during the study period in nutrient and control plots. Significant differences between treatment groups (SNK) are indicated with asterisks.

Nutrient addition stimulated leaf elongation in March and June 2004, but not in June 2003, immediately after the commencement of the experiment (see Fig. 9, Table 4a, SNK test $p< 0.05$). Mean annual growth rates were 0.63 ± 0.01 and 0.56 ± 0.01 cm shoot^{-1} day^{-1} in nutrient and control plots, respectively, with the highest values recorded in June. Growth was consistently higher in Zone A (coalescent patches) than in Zone B (isolated patches), with values of 0.64 ± 0.01 and 0.55 ± 0.01 cm shoot^{-1} day^{-1}, respectively. The influence of the sampling time varied to some extent between study zones (i.e. significant Month x Zone interaction) but, no significant differences were observed in the response of these two zones to nutrient enrichment.

ANOVA		**a) APP**				**b) Total Carbohydrates**		
Source of variation	**df**	**MS**	**F**	***p***	**df**	**MS**	**F**	***p***
Month = M	2	216.16	1035.2	**0.0000**	4	618.16	47.29	**0.0000**
Zone = Z	1	89.59	242.5	**0.0001**	1	9.33	0.26	0.6348
Treatment = T	1	38.08	100.6	**0.0006**	1	88.93	23.23	**0.0085**
Plot = P (T)	4	0.38	0.4	0.7954	4	3.83	0.73	0.5671
M x Z	2	3.06	14.8	**0.0020**	2	17.59	1.04	0.4156
M x T	2	10.33	49.5	**0.0000**	2	41.02	3.13	0.0439
Z x T	1	1.34	3.6	0.1294	1	6.94	0.19	0.6809
M x P (T)	8	0.21	0.2	0.9847	16	13.07	2.52	**0.0023**
Z x P (T)	4	0.37	0.4	0.8025	4	35.41	6.83	**0.0001**
T x P (T)	-	-	-	-	-	-	-	-
M x Z x T	2	0.29	1.4	0.2954	4	5.47	0.32	0.8575
M x Z x P (T)	8	0.21	0.2	0.9853	16	16.85	3.25	**0.0001**
M x T x P (T)	-	-	-	-	-	-	-	-
Z x T x P (T)	-	-	-	-	-	-	-	-
M x Z x T x P (T)	-	-	-	-	-	-	-	-
		Cochran's *C*: 0.208 (n.s) Transformation: -				Cochran's *C*: 0.079 (n.s) Transformation:-		

Table 4. Analysis of variance (ANOVA) on a) seagrass leaf growth and, b) total carbohydrate content in *Posidonia oceanica* (significant results are given in **bold**).

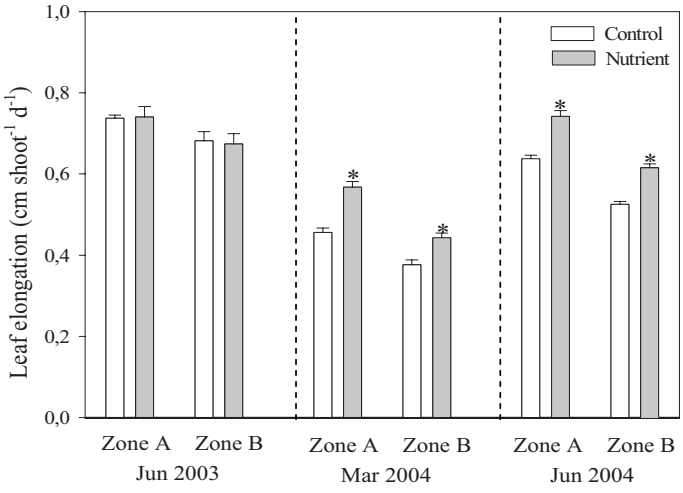

Fig. 9. Leaf elongationof *Posidonia oceanica* (cm^2 $shoot^{-1}$) at the three sampling times. Significant differences between treatment groups (SNK) are indicated with asterisks.

DISCUSSION

Sustained annual nutrient enrichment of *Posidonia oceanica* plots modified several features of the plant (e.g. increase in nutrient content) or of the epiphytic community (biomass, nutrient content, composition). Consequently, some key processes, including growth, herbivory or shading by epiphytes, were affected by nutrients with more or less intensity depending on the season (see fig. 10). Growth responded to the nutrient enrichment during the period of low nutrient availability (from spring to mid summer), herbivory during the period of high fish activity (from June to September) and shading by epiphytes did so in summer. In turn, alterations in such processes modified plant biomass and carbohydrate storage in a very variable way, depending mostly on their seasonality. Thus, in fertilized plots, while biomass increased in early spring, probably due to the direct response of growth to nutrient increase, it decreased in late summer, probably as a consequence of increased herbivory (see Fig. 10). The effects were similar at the two zones, patchy and continuous meadow, although leaf growth was consistently lower in the former. Macroalgal cover remained unaltered, possibly due to the relatively exposed conditions of the study site. In fact, blooms of macroalgae are most often found in sheltered sites, such as shallow bays

and estuaries or coastal lagoons with limited water exchange with the offshore circulation (e.g. Harlin & Thorne-Miller 1981, Lavery et al. 1991, Pedersen & Borum 1996, Valiela et al. 1997, McGlathery 2001).

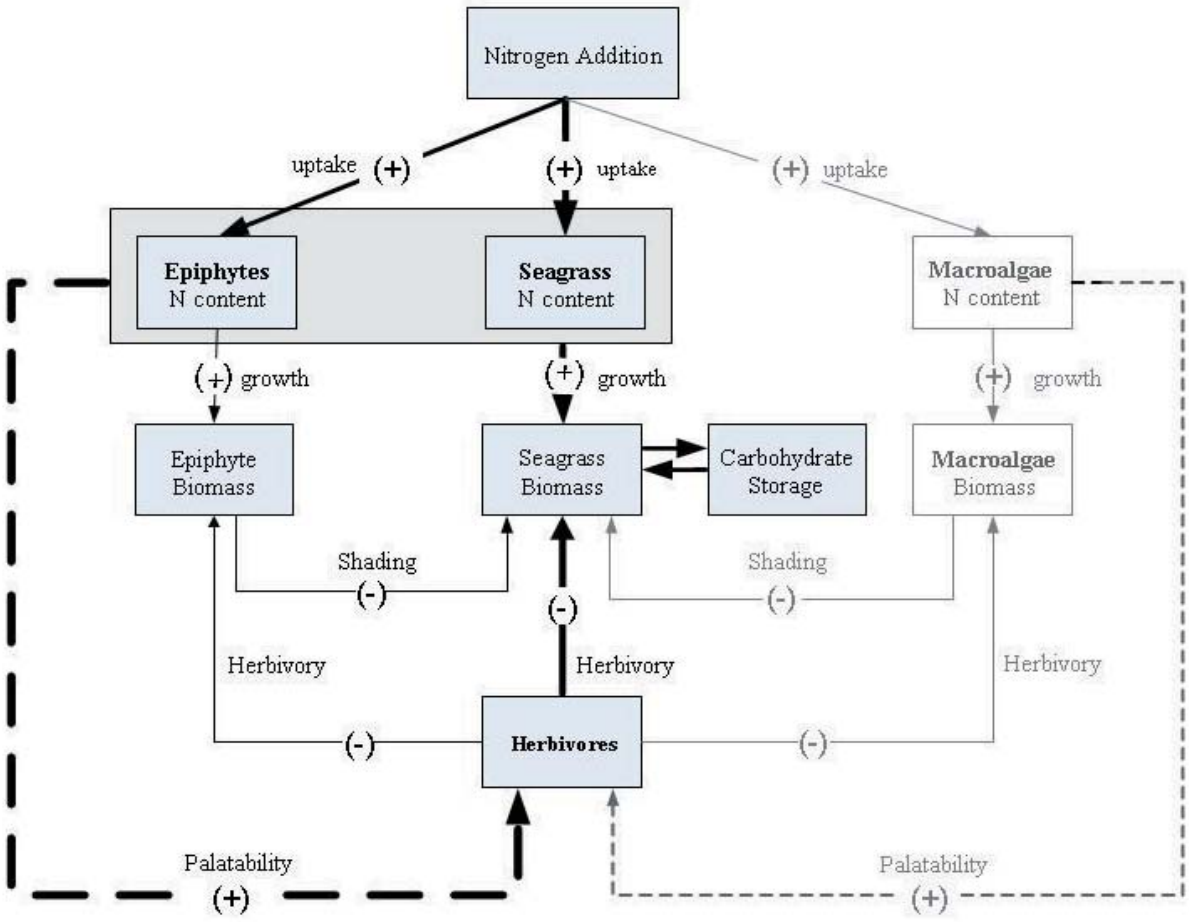

Fig. 10. Sequence of flow-on effects observed during the experimental period in the studied *Posidonia oceanica* meadow among all possible pathways of nutrient-induced changes.

Epiphyte biomass showed little response to nutrient addition except in summer (87% increase in August), indicating that epiphytic communities are more closely controlled by seasonal patterns of light availability than by nutrient availability (Alcoverro et al. 1997). Despite this biomass increase, shading by epiphytes caused only a modest decrease of the net summer plant carbon gain (6% of summer carbon gains, even less on an annual basis). This is due to the fact that most of their biomass was accumulated on the oldest parts of the leaves,

which have a lesser contribution to the plant C gains (Alcoverro et al. 2001). Besides, given that summer is the period with the lowest seasonal plant growth (Romero 1989, Alcoverro et al. 1995), the impacts of shading in plant biomass (e.g. in explaining biomass reduction in fertilized plots in summer) should be very limited, if any (Tomas et al. 2005b).

The herbivore pressure exerted by the fish *Sarpa salpa* was high in both treated and control plots throughout the summer (see also Tomas et al. 2005a, Prado et al. in press) but in the nutrient addition plots resulted on an earlier decreased leaf length during June-July attributable to an earlier plant consumption (lower number of intact leaves in such plots). This earlier plant leaf removal appeared in the period of maximum carbohydrate accumulation (i.e. June-July; see Alcoverro et al. 2001) and may account for the decrease in the rhizomes carbon reserves, as already observed by other authors (Zimmerman et al. 2001). However, direct effects of nutrients on the carbohydrates synthesis cannot be discarded (Invers et al. 2004). Biomass removal by herbivores could also have positive influences on leaf production (Valentine et al. 1997, Heck et al. 2000, Moran et al. 2002, Vergés et al. unpublished results) but no data on the summer period is available for shoot growth; in any case, this effect, if existing, was not observed on plant biomass.

The increase in fish activity in fertilised plots (relative to control ones) took place at the same moment in which differences between treated and untreated plots in nutrient content in both plant and epiphytes became apparent. Indeed, an increase in nitrogen content is believed to increase leaf palatability (sensu Mazzella et al. 1992). Moreover, this was also the time of the year in which changes in epiphyte composition due to the added nutrients occurs (Prado et al. in review), suggesting that the herbivores behaviour can be influenced, to some extent, by the epiphytic community composition. However, this situation was unexpectedly altered in mid summer (August-September) as fish performed foraging incursions in control plots that smoothed the differences in leaf length between treatments. This switch in fish behaviour can be caused by the aggregation of fishes and the limitation in the seagrass resource at the end of the foraging period, as the seagrass surface of the study area is small (low FHS, sensu Prado et al. ms in preparation) or by additional changes on epiphyte

composition between plots at the end of the summer (Prado et al. in review).

The quality of food has been shown to have a determinant influence of consumption rates for many herbivorous species (Shepherd 1987, Knoepffler-Peguy et al. 1987, Worm et al. 2000, Ruiz et al. 2001), including fish (Goecker et al. 2005). Numerous macroalgal species synthesize chemical and structural compounds that made them less palatable to consumers (Hay et al. 1987, Duffy & Hay 1990, Hay 1996, Cronin & Hay 1996) and as a result herbivory pressure could be partly controlled by species composition (Mazzella & Russo 1989, Gacia et al. 1999). In fact, species similar to *Sphacelaria cirrosa* – the dominant macroalgae present in fertilized plots during late summer (Prado et al. in review) – have been shown to be chemically defended against different groups of animals, including fish (Amsler et al. 2005) and could explain the herbivore shifts.

Leaf biomass increased in response to nutrient fertilization in early summer, most probably due to the direct effects of nutrients on leaf growth. This occurs when plant nutrient content declines, suggesting that the experimental nutrient supply alleviates nutrient shortage, confirming previous findings for the same season (Alcoverro et al. 1997). Later, these differences between treatments disappear (and reverse) due to fish action, which largely exceeds growth during that period (Tomas et al. 2005a, Prado et al. in press).

In summary, the plant biomass has been shown to substantially respond to changes in nutrient availability, although concurrent interactions between ecosystem processes made this response neither simple nor direct. Nutrients are often reported to stimulate plant primary production (Udy & Dennison 1997) or to decline plant vitality through increased epiphyte or macroalgal shading (Orth & Moore 1983, Cambridge & McComb 1984). Indirect effects mediated by herbivores (Gacia et al. 1999, this study) and landscape-level considerations (Prado et al. ms. in preparation) add further complexity to this scenario. A better understanding of eutrophication effects would probably need a careful assessment of such multi-level concurrent and flow-on interactions.

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