

Nitrogen load and irradiance affect morphology, photosynthesis and growth of *Caulerpa prolifera* (Bryopsidales: Chlorophyta)

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ABSTRACT: The effect of nitrogen (N) load and irradiance on morphology, growth and photosynthetic performance was studied in the green macroalga *Caulerpa prolifera* (Forsskål) J. V. Lamouroux from the Gulf of Cádiz (south Spain). Constant growth rates were obtained for thalli growing at different N loads, which could be fitted to tissue N using the Droop equation, rendering a maximum growth rate of 0.09 d⁻¹, a minimum tissue N level of 1.71 % dry weight (DW) and a critical tissue N of 5.2% DW. N limitation had no effects on F_v/F_m (maximum quantum yield of chlorophyll *a* fluorescence). Stolon production was significantly highest at low N loads; a reverse trend was observed for assimilator production. In a second experiment, algae were subjected to combinations of high and low N loads (HN and LN) and irradiance (HL and LL) levels. Highest growth rates were observed in the HNLL treatment, whereas the reverse combination rendered the lowest growth rate. High irradiance and high N load both led to increased biomass allocation to assimilators; at low N, the bulk of the biomass (>75 % in the HLLN treatment) was allocated to the stolons. HN had a positive effect on F_v/F_m , and HL had a negative effect. HL algae had a higher capacity for non-photochemical quenching. Despite its prolific nature, *C. prolifera* should be characterised as a slow-growing, but highly nitrophilic alga which has the capacity to forage for nutrients by allocating biomass to the stolons.

KEY WORDS: *Caulerpa prolifera* · Clonal growth · Morphology · Nitrogen foraging · Growth rate · Photosynthesis

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INTRODUCTION

Many higher plant species exhibit an architecture of clonal growth, i.e. a plant (genet) consists of units (ramets) which are repeated during growth. Ramets are interconnected through horizontal rhizomes and are further composed of a set of leaves (the shoot) and a root system. This clonal architecture has significant consequences with respect to life-history traits, competition, etc. (de Kroon & van Groenendaal 1997). Terrestrial plants can use their clonal structure to most efficiently locate and explore soil nutrient micropatches (Oborny & Cain 1997). By investing in new ramet pro-

duction or, alternatively, in rhizome expansion, clonal plants are able to deal with adverse conditions and can rapidly colonise a new environment.

Seagrasses all share this architecture of clonal growth (Hemminga & Duarte 2000). An important number of seaweeds likewise possess a clonal plant morphology, although its potential ecological consequences are rarely recognised and remain largely unexplored (Collado-Vides 2002a, Santelices 2004). With respect to resource acquisition, clonal growth appears to be particularly effective in a heterogeneous environment. As it is generally assumed that algae assimilate the majority of their nutrient requirements

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from the water column and are, hence, homogeneously distributed, the importance of clonal growth for resource acquisition seems less obvious. However, there is increasing evidence that a number of seaweeds can utilise detritus or sediment nutrient sources (Williams 1984, Larned 1998). Considering this, a clonal growth strategy might very well be feasible for seaweeds as well. Indeed, for a number of *Caulerpa* species, various authors have made implicit (Ceccherelli & Cinelli 1997) and explicit (Collado-Vides & Robledo 1999, Collado-Vides 2002b, Chisholm & Moulin 2003) suggestions that foraging might occur. However, experimental data on this subject are missing thus far (Collado-Vides 2002a, Santelices 2004).

The green algal genus *Caulerpa* J. V. Lamouroux (Bryopsidales: Chlorophyta) is a well-known inhabitant of (sub)tropical seas. It is a coenocyte, i.e. each individual consists of 1 giant (up to several decimetres in length), multinucleate cell. The form of the individuals is supported and maintained by numerous ingrowths of cell-wall material, the trabeculae (Jacobs 1994). Although great interspecific morphological variability exists, all species follow a common architectural scheme, consisting of a creeping stolon bearing various erect assimilators and rhizophores with rhizoid clusters (Collado-Vides 2002a, de Senerpont Domis et al. 2003). The rhizoids do not only function as a hold-fast, but are also involved in nutrient uptake from the sediment (Williams 1984, Chisholm et al. 1996). In this respect the rhizoids are analogous to the roots of vascular plants, whereas the stolons and the assimilators can be considered analogous to the rhizomes and the shoots of vascular plants.

Caulerpa prolifera is the only native *Caulerpa* species in continental Europe, occurring in the Mediterranean and the adjacent part of the Atlantic Ocean (Gulf of Cádiz). Additionally, *C. racemosa* and *C. taxifolia* have invaded the Mediterranean, causing a threat to the rich biodiversity of Mediterranean seagrass and seaweed stands (Meinesz et al. 2001, Piazzini et al. 2001). The 3 species form extensive beds and play an important role in ecosystem structure and functioning (Terrados & Ros 1991, Sánchez-Moyano et al. 2001). Despite this, data on *Caulerpa* nutritional ecology and ecophysiology are scarce and mainly related to nutrient uptake from the sediments (Williams 1984, Chisholm et al. 1996, Chisholm & Moulin 2003). Virtually nothing is known about the relation between nitrogen content and growth or the effect of nutrient loads on photosynthesis and algal architecture. This is ever more important given the recent debate on whether the invasive *Caulerpa* species form a higher risk in more eutrophic environments (Meinesz et al. 2001, Jaubert et al. 2003). The present paper was aimed to fill this gap by reporting the results of 2 experiments

with *C. prolifera* from the Bay of Cádiz (SW Spain) with the objectives (1) to test the hypothesis that the alga follows the strategy of clonally growing plants with respect to resource acquisition, (2) to determine the relationship between nitrogen content and growth rate and (3) to detect the effects of nitrogen availability and irradiance on photosynthetic performance.

MATERIALS AND METHODS

Algal collection and cultivation. For the first experiment, attached individuals of *Caulerpa prolifera* (Forskål) J. V. Lamouroux were collected from the mouth of the Río San Pedro tidal inlet near Puerto Real, southwest Spain (36°31'N, 6°14'E), in October 2001. The algae were cultured in aquaria filled with filtered (Whatman GF/C), nutrient-poor oceanic water at ambient temperatures (20 to 23°C) for 3 wk, in order to induce nutrient starvation. Light was provided in a 14 h light:10 h dark cycle by a combination of a white fluorescent tube (Narva daylight, 30 W) and a red fluorescent tube (Sylvania Gro-Lux, 30 W), resulting in a total underwater irradiance of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For the second experiment, algae were collected from the same site in June 2003. As storms had 'uprooted' the thalli, loosely floating individuals were collected. The experiment was started directly on the day of collection: 15 thalli, collected on the same date, were used to determine the relations between morphometry (stolon and assimilator length) and fresh (FW) and dry weight (DW).

Experimental set-up. Effects of nitrogen load on growth, photosynthesis and morphology: Algae were carefully divided in experimental units, each consisting of a stolon bearing 2 assimilators and a number of rhizoid clusters. The units were carefully blotted dry between 4 layers of tissue, weighed and their stolon length was recorded. At the beginning of the experiment, the units were divided into 8 Erlenmeyer flasks, leaving 2 units per beaker. The plants were colour-marked using a piece of thread. The Erlenmeyer flasks were filled with 1 l of artificial seawater (S = 35 psu) prepared from a sea salt mixture (hw Marinemix professional, Wiegandt, Germany). Salinity was checked using a hand refractometer (Atago). The Erlenmeyer flasks were placed in a tank filled with freshwater, which was connected to a thermostatic waterbath kept at 23°C. Light was provided by Philips TL20W/54RS fluorescent tubes, at an intensity of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under a 16 h light:8 h dark cycle. Light intensity was measured with a LI 193-SA (LiCor) quantum meter mounted with a 4 π PAR (400 to 700 nm) sensor. Nutrients (nitrogen and phosphate) were added from a stock solution containing 100 mM NH_4Cl and 4 mM

NaH₂PO₄·H₂O. By adjusting the addition volume, a range of 8 initial nutrient concentrations was obtained, which were converted to daily loads per gram fresh weight of algae (Table 1). Every 2 d all units were collected, cleaned of epiphytes (if necessary), carefully blotted dry between tissue, weighed and replaced into clean Erlenmeyer flasks with fresh medium. The addition volume was adapted to the new fresh weight in such a way that the nutrient load for each treatment was kept constant during the experiment. During the last 2 wk of the experiment, 47.8 µM GeO₂ was added to the medium to suppress diatom growth (Le Gall et al. 1990). The experiment lasted 32 d, after which all units were collected and weighed; the stolon length produced was recorded and the final number of assimilators was counted. Units were then split in half. One part was dried at 50°C for 1 wk to determine tissue C, N and P content. The other half was frozen in liquid nitrogen and stored at -80°C for later chlorophyll analyses. The specific growth rate (μ) was determined by calculating the slope of the line through the napierian logarithms (ln) of the fresh weight during the exponential growth phase, which started after a lag phase of 10 d to the end of the experiment.

Algal photosynthetic performance was assessed by calculating the maximum quantum yield of photosynthesis using measurements of chl *a* fluorescence (for details on this technique see Krause & Weis 1991, Schreiber et al. 1999). The measurements were made with a PAM-2000 (Walz) on Days 18, 26, 28 and 29 of the experiment, at various times to detect potential diurnal dynamics or effects of medium change. Of each of the units in each treatment, 2 assimilators were chosen haphazardly, resulting in 4 readings per Erlenmeyer flask. The assimilators were mounted with a dark leaf clip (handmade or Diving-LC, Walz). A 5 s weak farred pulse was administered to oxidise the electron transport chain (Hanelt 1998), after which the shutters of the clips were closed. After 5 min of dark acclimatisation, the base fluorescence F_0 was measured, followed by a saturating pulse to measure F_m , allowing calculation of the variable fluorescence ($F_v = F_m - F_0$) and the maximum quantum yield of PSII (Photosystem II) (F_v/F_m). The period of 5 min of dark acclimatisation after a 5 s far-red pulse was found to be sufficient to relax reversible non-photochemical quenching in various green macroalgal species (Hanelt 1998, Bischof et al. 2002).

Effects of irradiance and nutrient levels on growth, photosynthesis and morphology: Effects of nitrogen load, irradiance and their interaction were tested in a 2 × 2 fully factorial nested design (with plants nested in aquaria). Eight 30-l aquaria were filled with a layer of approximately 2 cm natural sediment and 20 l of artificial seawater (S = 35 psu, see above) and placed in a

Table 1. Initial nitrogen (N) and phosphorus (P) concentrations (µM) and loads (normalised to algal fresh weight, µmol nutrient g FW⁻¹) used in an experiment to determine the relationship between nutrient load and growth rate of *Caulerpa prolifera*

N		P	
Concentration	Load	Concentration	Load
1	1.0	0.25	0.25
5	4.9	1.25	1.22
10	14.0	2.50	3.50
15	22.6	3.75	5.66
20	31.5	5.00	7.87
30	54.4	7.50	13.60
40	57.0	10.00	14.26
50	82.3	12.50	20.58

cultivation chamber (D-1400-3BL, ASL). The sediment was washed thoroughly with 5 to 10% sulphuric acid and tap water to reduce organic material and nutrients. The water was gently aerated using airstones, and the temperature of the cultivation chamber was kept at 23°C. Light was provided by Philips TLD 18W/54 fluorescent tubes under a 16 h light:8 h dark cycle. The aquaria were subjected to a combination of either high (HL, 210 µmol photons m⁻² s⁻¹) or low light (LL, 98 µmol photons m⁻² s⁻¹) and high (HN, 25 µM N and 1 µM P) or low nutrients (LN, no additions), resulting in a total of 2 aquaria for each of the 4 treatments (HLHN, LLHN, HLLN and LLLN). Light level in the LL treatments was diminished by removing half of the fluorescent tubes. Nutrients were added from a stock solution (see above). The water was changed weekly.

Four thalli were incubated per aquarium, resulting in a total number of 8 thalli per treatment. Algal condition was determined to select the algae for the experiment. Photosynthetic performance, measured as F_v/F_m , was used as a condition parameter. Thalli, whose assimilators had a $F_v/F_m \geq 0.75$ were assumed to be in good condition (considering a maximum F_v/F_m of 0.80; Häder et al. 1997, Carr & Björk 2003) and were selected for the experiment. Selected thalli were colour-coded using a piece of thread. For each thallus, stolon length was recorded, the number of assimilators counted and their length measured. Additionally, the number of proliferations was counted and their length was measured as well. Algae were carefully blotted dry between 4 layers of tissue and weighed. The thalli were evenly distributed in each of the aquaria, gently pushing their rhizoids into the sediment. After 4 wk the experiment was terminated. Photosynthetic efficiency was assessed by F_v/F_m as described for the first experiment. Additionally, photosynthesis (as relative electron transport rate, rETR) versus irradiance curves were determined as rapid light curves (RLCs, e.g. Carr &

Björk 2003). Assimilators were mounted in the dark leaf clips and were subjected to a series of 10 increasing PAR (photosynthetically active radiation) intensities, ranging from 44 to 2004 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, using the halogen lamp from the PAM as the light source. Light levels were measured with a LI 193-SA (LiCor) quantum meter mounted with a 2 π PAR (400 to 700 nm) sensor. After 30 s of exposure time to each light intensity, the steady-state fluorescence (F_i) was measured, followed by a saturating light pulse to measure the maximum fluorescence (F_m'), and the effective quantum yield of PSII [$\Delta F/F_m' = (F_m' - F_i)/F_m'$] was calculated. rETR was calculated as $\text{rETR} = \Delta F/F_m' \times \text{PAR}$. Additionally, the coefficients for photochemical (q_p) and non-photochemical quenching (q_N) were calculated (Schreiber et al. 1999). Then, rETR was plotted versus PAR. As *Caulerpa prolifera* showed no signs of photoinhibition, the photosynthesis parameters maximum rETR (rETR_m) and efficiency (α) were estimated by fitting the data to the model of Jassby & Platt (1976) using least-squares non-linear regression. The onset of light saturation (I_k) was then calculated as rETR_m/α . Finally, the thalli were carefully blotted dry and weighed. Relative daily growth rate (RGR) was determined as:

$$\text{RGR} = \frac{\ln(W_t - W_0)}{t}$$

where W_t and W_0 are final and initial fresh weight and t is time (28 d). The same morphometric measurements were made as at the beginning of the experiment, after which plants were split in 2 for analyses as in the first experiment.

Chemical analyses. Oven-dried plants were ground using a mortar and pestle. Tissue C and N content were determined on a Perkin-Elmer 240 C elemental CHN analyser. Phosphorus content was measured colourimetrically following Grasshof et al. (1983) after persulphate digestion in an autoclave. Frozen plants were ground in liquid nitrogen with a mortar and pestle. Chlorophyll *a* and *b* concentrations were determined after overnight extraction in acetone (Jeffrey & Humphrey 1975).

Data analysis. Specific growth rates (μ) from the first experiment were plotted against tissue N content (N), and the data were fitted to the Droop equation (Droop 1968), using non-linear least-squares regression:

$$\mu = \mu_{\max} \left(1 - \frac{N_Q}{N} \right)$$

where μ_{\max} represents maximum specific growth rate, and N_Q , the subsistence nitrogen quota (minimum N tissue concentration needed before growth can proceed). A third parameter estimated was N_C , the critical nitrogen concentration needed to sustain growth at maximum rates (sensu Pedersen & Borum 1997). N_C was estimated as the intercept between the initial slope of the Droop curve and μ_{\max} (Pedersen & Borum 1997). For comparison, a linear regression was also performed with tissue N as the independent and μ as the dependent variable.

For Expt 2, differing investments in assimilators or stolons were analysed. The best way to do this is to analyse biomass partitioning for which separate weight determinations for both proportions are necessary. Given the fact that *Caulerpa prolifera* is coenocytic and will, therefore, lose parts of its cellular contents when damaged, probably resulting in a significant underestimation of biomass, we decided not to adopt such a destructive approach. Instead, biomass was derived from stolon and assimilator lengths, using the morphometric relations determined on plants sampled at the same time as those used in the experiment. As stolons have a cylindrical morphology and stolon thickness appeared more or less constant, stolon weight can be expected to vary linearly with stolon length. Linear regression showed that this was indeed the case, and a conversion factor was calculated by fitting a linear model with the data, forcing the intercept through zero (Table 2). For assimilators the relation between length and weight is less straightforward, as they start heart-shaped or circular and become slightly more elliptic. Hence, weight can be expected to be linearly related to the squared length in short assimilators and to length in long assimilators. A linear relationship with squared length was found up to an assimilator

Table 2. *Caulerpa prolifera*. Results of linear regression (intercepts forced through zero) between stolon lengths (mm) and fresh weight (FW, mg) and assimilator lengths squared (mm^2) and FW (mg) and between calculated and measured FW of algae (mg) at the beginning and end of Expt 2 (see 'Materials and methods' for explanation). Significant correlations are denoted:

*** $p < 0.001$

Independent	Dependent	R ²	Slope	Intercept
Stolon length	Stolon FW	0.78***	1.14	0.00
Assimilator length, squared	Assimilator FW	0.85***	0.02	0.00
Calculated initial plant FW	Measured initial plant FW	0.76***	0.70	0.02
Calculated final plant FW	Measured final plant FW	0.88***	0.91	-0.05

length of 60 to 70 mm, after which marked deviations from linearity occurred. As only 4 assimilators (5.2% of the total in initial plants) were longer than 65 mm, this was not considered a problem, and a conversion factor was calculated for squared assimilator lengths to assimilator weight by linear regression (Table 2). For each plant, total initial and final fresh weight were then calculated as:

$$W = \sum_{i=1}^n (1.142 \times S_i) + \sum_{j=1}^m (0.0215 \times A_j^2)$$

where W is weight (mg), S is stolon length (mm) and A is assimilator length (mm) for n stolons and m assimilators per plant (initial plants all had only 1 stolon, but after 4 wk several plants had formed new stolons). A linear regression performed on calculated weights versus observed weights gave satisfactory results for both initial and final plants, validating our approach (Table 2).

The effects of nutrients and light and their interactions on RGR, plant morphology (see Table 3 for list of characters analysed), F_v/F_m and RLC parameters ($rETR_m$, α and I_k) were tested using a nested 2-way ANOVA, followed by a Tukey honestly significant difference test for unequal sample sizes (Sokal & Rohlf 1995). Data were tested for heterogeneity of variances with Bartlett's test for homogeneity prior to the ANOVA. Assimilator density (#A/S) was log-transformed, which removed heterogeneity. No transformation could be found which removed the heterogeneity from the quantum yield data. Finally, a 2-way ANOVA was performed on the transformation that was most effective in reducing the heterogeneity of variances ($p \approx 0.01$ in Bartlett's test for homogeneity): $y' = 1/\ln y$.

Table 3. *Caulerpa prolifera*. Morphological characteristics measured and their abbreviations

Character code	Description
mm Stol	Total stolon length produced per plant (final – initial)
mm Ass	Total assimilator length produced per plant (final – initial)
#Stol	Number of stolons produced per plant (final – initial)
#Ass	Number of assimilators produced per plant (final – initial)
#Prol	Number of proliferations produced per plant (final – initial)
#A/S	Number of assimilators per centimetre stolon (final plants)
Avg Ass	Average assimilator length per plant (final plants only)
%Ass	Percent of biomass allocated to assimilators (final plants only)

The design was relatively large (4 treatments nested design, $n \geq 6$), however, with unequal sample size, so the results have to be treated with care (Underwood 1997). In the 'Results' and the 'Discussion' sections, the causes of this heterogeneity are analysed. To detect the potential effects of treatments on assimilator size distribution of the entire population of assimilators (independent of the plants to which they belong), the sizes of all assimilators were pooled per treatment and tested pairwise using Kolmogorov-Smirnov 2-sample tests (Sokal & Rohlf 1995).

RESULTS

Effect of nitrogen load on growth, photosynthesis and morphology

Thalli cultured at an initial concentration of 20 $\mu\text{M N}$ died after 2 wk for unknown reasons. The other thalli grew well, obtaining constant growth rates after 7 to 10 d. At higher concentrations ($\geq 30 \mu\text{M N}$ initial) epiphytes (mainly small rhodophyte macrophytes) developed that were removed frequently and did not seem to impair the growth rate. The range obtained in tissue N concentrations after the starvation and experimental period was rather small: 2.3 to 4.7% DW. Nevertheless, a clear increase in growth rates with tissue N concentration was observed (Fig. 1). A linear regression returned the equation $\mu = 0.018 \times N - 0.0165$, with $R^2 = 0.69$ ($F = 29.15$, $p < 0.001$, $n = 14$). The Droop equation gave very similar R^2 (0.66) and ANOVA results ($F = 27.9$, $p < 0.001$, $n = 14$) and estimates \pm standard errors for μ_{max} ($0.09 \pm 0.01 \text{ d}^{-1}$) and N_Q ($1.71 \pm 0.14 \%$ DW), from which N_C was calculated (5.2% DW). Thallus morphology showed a marked response to nitrogen load. A clear tendency for an increasing number of

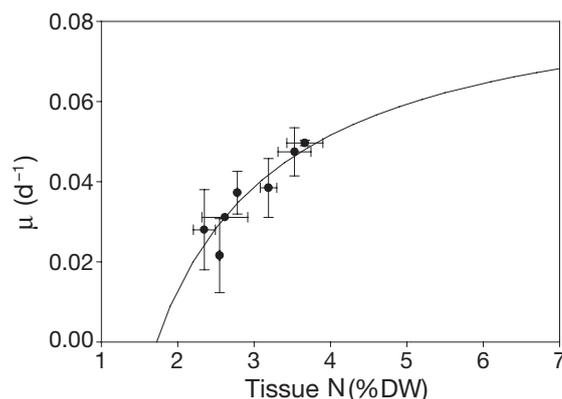


Fig. 1. *Caulerpa prolifera*. Relation between specific growth rate (μ , d^{-1}) and tissue nitrogen content (% dry weight) fitted to the Droop equation (continuous line). Error bars represent ± 1 SD

assimilators at higher nitrogen loads was observed, whereas stolon production showed the reverse trend (Fig. 2A). In addition, it has clearly been observed that algae at the lowest loads produced more and longer rhizoids; unfortunately, we did not quantify this. F_v/F_m did not vary with N load (Fig. 2B), nor was there significant variation in quantum yield during the day or between days within treatments (data not shown).

Effects of different light and nutrient levels on growth and morphology

Most plants grew well in the aquaria, developing new assimilators and stolons already during the first week of incubation. No development of epiphytes or excessive microalgal growth occurred. There was high variation in growth rates, morphology, chlorophyll content and photosynthetic performance between plants and within treatments. Nevertheless, clear and significant responses could be observed. Tissue N and P were higher in assimilators than in stolons of initial plants (Fig. 3). Tissue N contents were highest in ini-

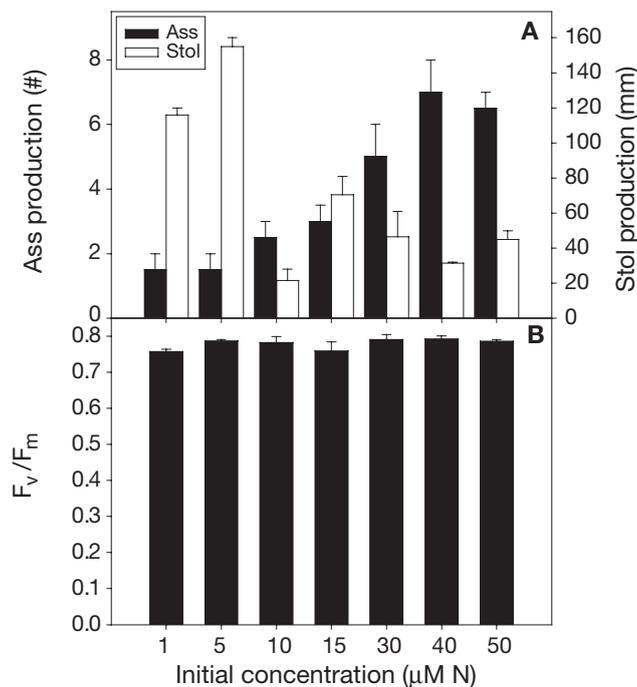


Fig. 2. *Caulerpa prolifera*. Effect of increasing nitrogen loads (given as initial nitrogen concentration, $\mu\text{M N}$) on (A) morphology and (B) maximum quantum yield (F_v/F_m) of photosynthesis. Morphological features measured were average number of assimilators (Ass) produced per plant and average stolon (Stol) length produced per thallus (mm). There were no significant differences between treatments ($p < 0.05$). Error bars represent ± 1 SD

tial plants compared to final plants, regardless of the experimental conditions (Fig. 3). Nutrient addition resulted in significantly higher tissue N and P levels, which were not affected by the light level (Fig. 3, Table 4). Tissue C contents were slightly smaller in the LLHN treatment compared to the other treatments, the difference was insignificant however (Fig. 3, Table 4). No significant differences were observed in pigment contents (data not shown). Variation was high in both high-light treatments.

Growth rates were generally low and were significantly influenced by both light and nutrient level (Fig. 4A, Table 5), but not by their interaction. The HLLN treatment was least favourable, even showing a biomass decrease. Surprisingly, the LLHN treatment had a higher RGR than the HLHN treatment, although the difference was not significant. Most of the biomass increase (or indeed decrease) was due to changes in assimilator size and number; whereas increases in stolon length did not differ significantly between treatments (Fig. 4B,C). Number of stolons, however, was

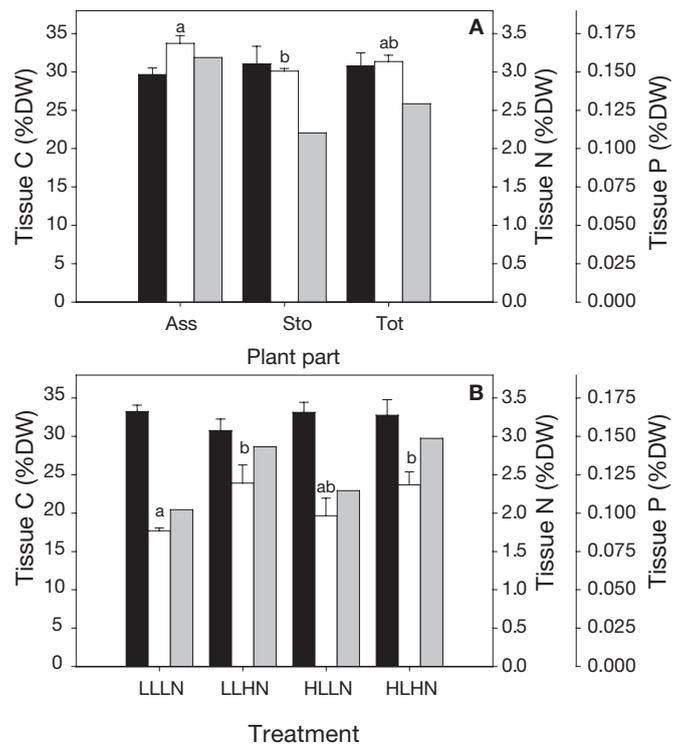


Fig. 3. *Caulerpa prolifera*. Tissue carbon (closed bars), nitrogen (open bars) and phosphorus (grey bars) levels (% dry weight) in algae (A) before and (B) after being subjected to combinations of low and high irradiance (LL and HL) and low and high nutrient load (LN and HN) (% dry weight). Nutrient levels in initial plants in assimilators (Ass), stolons (Sto) and whole plant levels (Tot). Different letters above bars indicate significant differences between treatments ($p < 0.05$). Error bars represent ± 1 SD

Table 4. Results of 2-way ANOVA on the effects of nutrient load (NUTR), irradiance (LIGHT) and their interaction (N × L) on tissue nutrient and chlorophyll contents of *Caulerpa prolifera* (italics significant at $p < 0.05$)

Variable	Factors	df	F-ratio	p
Tissue C	NUTR	1	1.867	0.244
	LIGHT	1	0.784	0.426
	N × L	1	0.990	0.376
Tissue N	<i>NUTR</i>	1	<i>14.950</i>	<i>0.018</i>
	LIGHT	1	0.420	0.552
	N × L	1	0.695	0.451
Chl a	NUTR	1	0.177	0.695
	LIGHT	1	0.010	0.927
	N × L	1	0.125	0.741
Chl b	NUTR	1	0.586	0.487
	LIGHT	1	0.027	0.878
	N × L	1	0.090	0.779

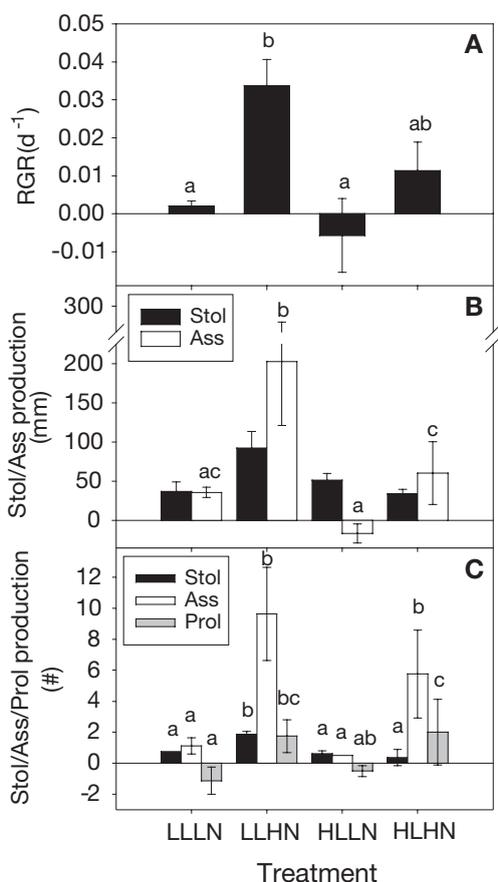


Fig. 4. *Caulerpa prolifera*. Results of combinations of low and high irradiance (LL and HL) and low and high nutrient load (LN and HN) on (A) relative growth rates (RGR, d^{-1}), (B) average stolon (Stol) and assessor (Ass) length produced per thallus (mm) and (C) average number of stolons, assimilators and proliferations (Prol) produced per thallus. Different letters above bars indicate significant differences between treatments ($p < 0.05$). Error bars represent ± 1 SD

significantly higher in the LLHN treatment, with no differences between the other treatments (Fig. 4C, Table 5). It can be clearly seen that the negative RGR and the decrease in total assessor length in the HLLN treatments was due to losses in the number of proliferations (Fig. 4C). Indeed, loosely lying proliferations were found in these aquaria, which were in the process of decaying. Even higher losses of proliferations were observed in the LLLN treatment, but this was compensated by increases in assessor size and number (Fig. 4B,C).

Both light and nitrogen levels significantly influenced plant morphology. At the beginning of the experiment, there were no significant differences between treatments in assessor density, biomass allocation, or average assessor size, although variation

Table 5. Results of nested 2-way ANOVA on the effects of nutrient load (NUTR), irradiance (LIGHT) and their interaction (N × L) on growth rate and morphological characteristics of *Caulerpa prolifera* (italics significant at $p < 0.05$)

Variable	Factors	df	F-ratio	p
μ	Nest	4	0.593	0.672
	<i>NUTR</i>	1	<i>14.120</i>	<i>0.001</i>
	<i>LIGHT</i>	1	<i>5.390</i>	<i>0.030</i>
	N × L	1	1.266	0.273
mm Stol	Nest	4	0.190	0.941
	NUTR	1	0.762	0.391
	LIGHT	1	1.047	0.316
	N × L	1	2.889	0.102
mm Ass	Nest	4	3.443	0.023
	<i>NUTR</i>	1	<i>48.318</i>	<i><0.001</i>
	<i>LIGHT</i>	1	<i>30.718</i>	<i><0.001</i>
	N × L	1	6.597	0.017
#Stol	Nest	4	0.943	0.456
	NUTR	1	4.200	0.052
	<i>LIGHT</i>	1	<i>14.486</i>	<i>0.001</i>
	N × L	1	10.371	0.004
#Ass	Nest	4	1.682	0.187
	<i>NUTR</i>	1	<i>36.741</i>	<i><0.001</i>
	LIGHT	1	3.935	0.059
	N × L	1	2.053	0.165
#Prol	Nest	4	2.297	0.088
	<i>NUTR</i>	1	<i>20.319</i>	<i><0.001</i>
	LIGHT	1	0.538	0.470
	N × L	1	0.099	0.756
% Ass	Nest	4	1.002	0.426
	<i>NUTR</i>	1	<i>54.033</i>	<i><0.001</i>
	<i>LIGHT</i>	1	<i>29.426</i>	<i><0.001</i>
	N × L	1	1.466	0.238
#A/S	Nest	4	0.569	0.688
	<i>NUTR</i>	1	<i>39.014</i>	<i><0.001</i>
	LIGHT	1	3.936	0.059
	N × L	1	0.645	0.430
Avg Ass	Nest	4	0.210	0.931
	NUTR	1	2.817	0.106
	<i>LIGHT</i>	1	<i>18.412</i>	<i><0.001</i>
	N × L	1	0.800	0.380

was high in some cases (Fig. 5). During the experiment, assimilator density and size decreased in all treatments (Fig. 5A,C). High nutrient levels resulted in a higher density, independent of light level (Fig. 5A, Table 5). However, when biomass allocation is considered, both high light and high nutrient levels significantly had a higher proportion of the biomass allocated to assimilators independently of each other (no interaction, Table 5). The discrepancy between these 2 can be explained by the proliferations, which were higher in the high-nutrient treatments. In the HLLN treatment even more than 75% of the biomass was allocated to the stolons (Fig. 5B). Assimilator size was significantly higher at low light levels, independent of nutrients. This difference was confirmed in a comparison of the

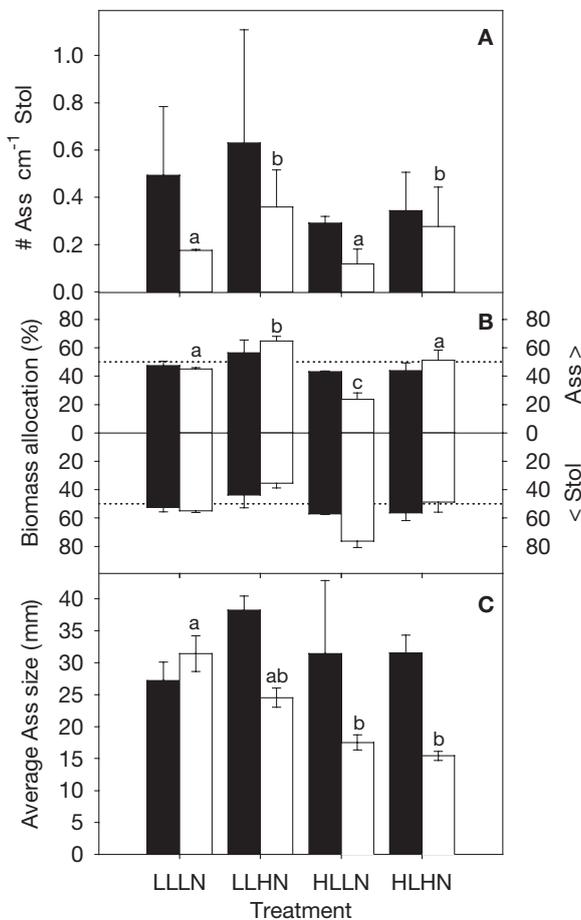


Fig. 5. *Caulerpa prolifera*. Results of combinations of low and high irradiance (LL and HL) and low and high nutrient load (LN and HN) on (A) assimilator density per stolon (# Ass cm⁻¹ Stol), (B) percent biomass allocated to stolons or assimilators and (C) average assimilator size per thallus (mm), before (closed bars) and after (open bars) the experiment. Dotted lines in (B) indicate 50% biomass allocation. Different letters above bars indicate significant differences between treatments ($p < 0.05$). Error bars represent ± 1 SD

size distribution of the entire assimilator population, where low-light size distributions significantly differed from high-light size distributions (Table 6). Assimilators from low-light algae could be found in all size classes, except the smallest, whereas assimilators from plants grown under high light were mainly clumped in the 4 smallest size classes (Fig. 6).

F_v/F_m was significantly affected by light and nutrients, but not by their interaction (Fig. 7, Table 7). The high-nutrient treatments had the highest efficiencies (highest in LLHN), whereas HLLN scored lowest (Fig. 7), with yields under 0.6, indicating photoinhibition. A significant effect of nesting (difference between plants within aquaria) and significant heterogeneity of variances were detected however; hence, the results have to be treated with care. Standard deviation was negatively correlated to average yield; apparently, as conditions become less optimal or more adverse for algal photosynthesis, the variation increases, both between individuals as well as between assimilators within individuals. RLCs of the plants fitted well to a production model excluding photoinhibition (>95% of

Table 6. Kolmogorov-Smirnov 2-sample test results (p) of pairwise comparisons of combinations of high and low irradiance (HL and LL, respectively) and high and low nutrient load (HN and LN, respectively) on the assimilator size distribution of *Caulerpa prolifera*

	LLLN	LLHN	HLLN	HLHN
LLLN	-	>0.10	<0.025	<0.001
LLHN		-	<0.10	<0.01
HLLN			-	>0.10

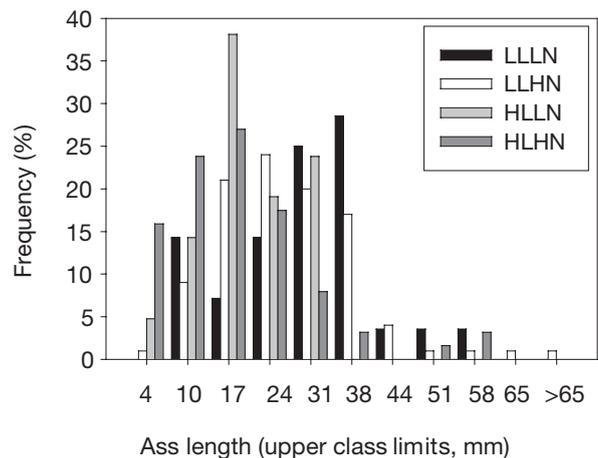


Fig. 6. *Caulerpa prolifera*. Frequency distribution of assimilator lengths (mm) after being subjected to combinations of low and high irradiance (LL and HL) and low and high nutrient load (LN and HN)

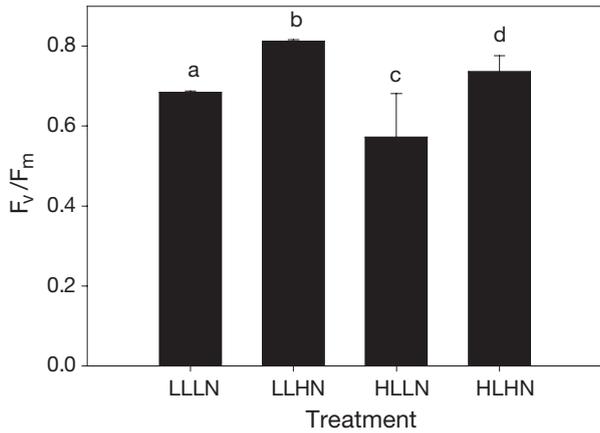


Fig. 7. *Caulerpa prolifera*. Results of combinations of low and high irradiance (LL and HL) and low and high nutrient load (LN and HN) on maximum quantum yield (F_v/F_m). Different letters above bars indicate significant differences between treatments ($p < 0.05$). Error bars represent +1 SD

the variation could be explained by the model in 36 of the 38 RLCs made, RLCs not shown). The RLC parameters $rETR_m$ and α significantly increased with increasing nutrients, but decreased with increasing light; hence, they were highest in the LLHN algae (Tables 7 & 8). It has to be kept in mind however that these are relative values, depending on algal light absorbance, which was unknown, but most probably different, at least between the HL and LL treatments. I_k was highest in the HN treatments and independent of light level. This parameter does not depend on absorbance.

Table 7. Results of nested 2-way ANOVA on the effects of nutrient load (NUTR), irradiance (LIGHT) and their interaction (N \times L) on maximum quantum yield of photosynthesis (F_v/F_m) and the *P-I* curve parameters $rETR_{max}$, α and I_k of *Caulerpa prolifera* (italics significant at $p < 0.05$)

Variable	Factors	df	F-ratio	p
F_v/F_m	<i>Nest</i>	4	6.371	0.010
	<i>NUTR</i>	1	103.078	<0.001
	<i>LIGHT</i>	1	43.964	<0.001
	<i>N \times L</i>	1	1.625	0.072
$rETR_{max}$	<i>Nest</i>	4	1.562	0.214
	<i>NUTR</i>	1	20.535	<0.001
	<i>LIGHT</i>	1	10.695	0.003
	<i>N \times L</i>	1	1.963	0.173
α	<i>Nest</i>	4	1.482	0.236
	<i>NUTR</i>	1	25.037	<0.001
	<i>LIGHT</i>	1	15.355	0.001
	<i>N \times L</i>	1	1.413	0.245
I_k	<i>Nest</i>	4	0.547	0.703
	<i>NUTR</i>	1	4.630	0.041
	<i>LIGHT</i>	1	0.380	0.543
	<i>N \times L</i>	1	0.005	0.942

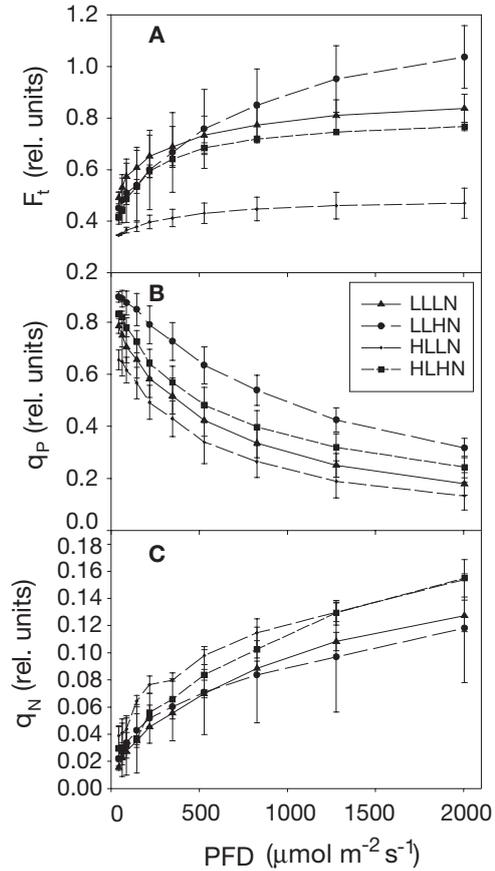


Fig. 8. *Caulerpa prolifera*. Results of combinations of low and high irradiance (LL and HL) and low and high nutrient load (LN and HN) on (A) steady-state fluorescence (F_t), (B) photochemical quenching (q_p) and (C) non-photochemical quenching (q_n). Error bars represent ± 1 SD

Clear responses to treatments were observed in the steady-state fluorescence (F_t) and quenching parameters (q_p and q_n) (Fig. 8). Acclimatisation to the cultivation light level clearly played an important part in determining F_t and q_n , as excess energy in plants grown under low light was mainly channelled away as

Table 8. *Caulerpa prolifera*. Average estimates (± 1 SD) of $rETR_m$, α and I_k from rapid light curves fitted to the equation of Jassby & Platt (1976) for plants subjected to 4 experimental treatments consisting of combinations of high and low irradiance (HL and LL, respectively) and high and low nutrient load (HN and LN, respectively). Different letters next to values indicate significant differences ($p < 0.05$) between treatments

Treatment	$rETR_m$ (rel. units)	α (rel. units)	I_k ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
LLLN	185.5 \pm 65.2 ^a	0.34 \pm 0.06 ^a	518.7 \pm 85.9 ^a
LLHN	397.7 \pm 34.7 ^b	0.53 \pm 0.07 ^b	753.7 \pm 23.8 ^b
HLLN	118.6 \pm 16.7 ^a	0.25 \pm 0.04 ^a	461.3 \pm 19.5 ^a
HLHN	230.7 \pm 85.8 ^a	0.37 \pm 0.01 ^a	680.8 \pm 176.0 ^b

F_v , while in high-light plants q_N played a more important role both in high- and low-nutrient regimes (Fig. 8A,C). Photochemical quenching (q_p) followed the same pattern as the RGR, rETR and the $P-I$ curve parameters in that it was highest at all light intensities for the LLHN plants and lowest for the HLLN plants (Fig. 8B).

DISCUSSION

Effect of nutrient availability on morphology

Clonal growth strategy for terrestrial plants predicts that clonal plants acclimatise their architecture to optimal foraging provided the habitat is temporally predictable (de Kroon & Hutchings 1995). This means that plants growing in nutrient-poor environments will allocate their resources to the development of below-ground biomass, consequently resulting in a rather low shoot/root biomass ratio (de Kroon & Hutchings 1995). Aquatic environments present a fundamental difference compared with the terrestrial environment, in that nutrients are both available in the water column and in the sediment. Hence, leaves of aquatic plants (or fronds of macroalgae) are 'multifunctional', i.e. are responsible for both photosynthesis and nutrient uptake. Although the water column is considered to be the major nutrient source for macroalgae, it has been shown that the sediment plays a much more important role as a nutrient resource than has generally been assumed (Williams 1984, Larned 1998). This means that the advantages of clonal growth with respect to nutrient acquisition also hold for aquatic plants, especially for seagrasses and macroalgae growing on sediments. Thus, at low nutrient levels, in the aquatic realm it can be expected that plants invest in roots and rhizomes or rhizoids to explore the sediment for resources. Indeed, this strategy has been reported for a number of seagrass species (Short 1983, Terrados et al. 1999).

In the present study, a strong morphological response to nutrient availability was recorded for the clonal-growing macroalga *Caulerpa prolifera*. Biomass allocation clearly shifted from stolons at low N loads to assimilators at high N loads, resulting in more and longer assimilators at higher loads, while development of stolons and rhizoids is stimulated in algae suffering chronic nutrient limitation. From this we conclude that *C. prolifera* forages and thus follows the strategy of clonally growing plants, for which experimental evidence has thus far been lacking (Collado-Vides 2002a, Santelices 2004). This is even more remarkable considering that *Caulerpa* is a coenocytic alga. The formation of extra assimilators and/or stolons or rhizoids is hence

merely a consequence of small-scale differences in cell-wall stretching. It is known that gravity and light climate are important factors in assimilator and rhizoid formation (Collado-Vides 2002b, de Senerpont Domis et al. 2003). The present results indicate that nutrient availability also has its effect on morphogenesis. Another remarkable response is the shedding of proliferations at low nutrient concentrations. It can be hypothesised that shedding is a functional response to nutrient limitation, to promote dispersal and hence secure the survival of the genotype. This, however, remains speculative.

Clonal land and aquatic plants growing under reduced light levels generally allocate their biomass to shoots and leaves, resulting in plants with a more compact growth form (de Kroon & Hutchings 1995). *Caulerpa prolifera* in this study indeed showed a biomass allocation pattern as described above. Algae grown at low light had more biomass allocated to assimilators compared to plants grown at the same nutrient level, but at a higher light level. Assimilator length was also clearly greater in low-light plants, which is in agreement with results of Collado-Vides (2002b). Growth rates, however, indicate that light was not limiting, on the contrary even, growth was highest at the low irradiance level. More experimental research, subjecting plants to a broader range of light levels, is needed to clarify whether biomass allocation in *C. prolifera* is influenced by light intensity.

Caulerpa growth rates and nitrogen requirements

Specific growth rates of *Caulerpa prolifera* in both experiments were low, never exceeding 0.05 d^{-1} . We did not find reports on μ of *C. prolifera* in the literature; however, Williams et al. (1985) and Larned (1998) reported μ in 5 other *Caulerpa* species to range between 0.014 and 0.073 d^{-1} . From the field biomass data of *C. prolifera* and *C. taxifolia* (Terrados & Ros 1991, Meinesz et al. 1995), net maximum growth rates can be calculated to range between 0.005 and 0.017 d^{-1} . Although these reported values cannot be compared directly, as they were recorded under different circumstances (different experiments, field data, etc.), they clearly indicate that, despite the rapid spreading of these species and the invasive behaviour of *C. racemosa* and *C. taxifolia* in the Mediterranean and other areas (Jousson et al. 2000, Schaffelke et al. 2002, Williams & Grosholz 2002), it can be concluded that *Caulerpa* spp. in general are relatively slow-growing species. In this respect they are comparable to seagrasses such as *Cymodocea nodosa* and *Zostera marina* (Hemminga & Duarte 2000) or slow-growing seaweeds (growth rates of various species listed in Fortes & Lüning 1980, Larned 1998).

This is in contrast to other proliferating green macroalgae such as *Ulva*, *Enteromorpha* and *Cladophora* species, which can have growth rates up to 0.3–0.5 d⁻¹ (Hernández et al. 1997, Malta & Verschuure 1997).

The low maximum growth rate predicted by the Droop equation confirms the characterisation of *Caulerpa prolifera* as a slow-growing alga. This estimate must be considered with some care however, as the point of saturation of growth with increasing tissue N content has not yet been observed and linear regression gave a similar value for R² as the Droop model. Nevertheless, the results clearly indicate that *Caulerpa* is a highly nitrophilic alga, as both the N_Q and N_C values are among the highest that have been determined for macroalgae (Pedersen & Borum 1996, Table 5.9 in Lobban & Harrison 1997, Campbell 2001). Contrastingly, algae with similar minimum N contents are all fast-growing species. High tissue N levels in *Caulerpa* species (up to 12.7% DW in *C. sertularioides*) have been reported before (Delgado et al. 1996, Robledo & Pelegrín 1997, Larned 1998). *Caulerpa* species are known to contain relatively high numbers of endosymbiotic bacteria (especially in the rhizomes, Chisholm et al. 1996, Meusnier et al. 2001), and marine bacteria generally have high nitrogen levels and C:N ratios equal to or even lower than the Redfield ratio for phytoplankton (Fukuda et al. 1998). Chisholm et al. (1996) estimated *C. taxifolia* to contain 10⁴ to 10⁵ bacteria mm⁻³. Assuming a N content of 5.8 fg cell⁻¹ (average for coastal bacteria, Fukuda et al. 1998) and a specific stolon dry weight of 6.34 × 10⁻⁵ g mm⁻³ stolon (taken from our own data on *C. prolifera*), the amount of bacterial N can roughly be estimated to range between 9.15 × 10⁻⁵ and 9.15 × 10⁻⁴% of *Caulerpa* dry weight. This means that, at the most, 0.05% of the measured N in *Caulerpa* is in fact bacterial N, a negligible amount.

The extensive secondary metabolism of *Caulerpa* species may be a better explanation of their high N requirements. *Caulerpa* species contain high levels of terpenes (up to 3.6–4.2% DW, Jung et al. 2002), which are thought to function as herbivore repellents and natural antibiotics (Paul & Fenical 1986, Jung et al. 2002). When wounded or attacked, a very rapid enzyme-mediated conversion of these terpenes occurs, enhancing the chemical protection of the alga even more (Jung et al. 2002). Caulerpenyne and the conversion products do not contain nitrogen; however, this fast response implies that the enzymes needed for the transformation should be present (and active) in relatively high amounts. Shanmugam et al. (2001) indeed have reported high protein levels in various *Caulerpa* species; however, Robledo & Pelegrín (1997) found relatively low amounts in *C. racemosa*. Besides terpenes, *Caulerpa* spp. also possess secondary metabolites that

do contain nitrogen (see e.g. Faulkner 1999, Levi & Freidlander 2004). However, other explanations for the high nitrogen requirements of *Caulerpa* spp. may exist, and further studies on this aspect are needed.

Despite the high N requirements, many studies have demonstrated the lack of *in situ* nutrient limitation in *Caulerpa* spp., even in nutrient-poor environments (Delgado et al. 1996, Ceccherelli & Sechi 2002). The algae have high nutrient-uptake capacities from the sediment through their rhizoids (Williams 1984, Chisholm et al. 1996); according to Williams et al. (1985) sediment uptake even accounts for the total N requirement. Additionally, studies on *C. taxifolia*-inhabited sediments show a stimulation of the breakdown of organic material and of bacterial nitrogen fixation (Chisholm & Moulin 2003), thereby increasing the sediment nutrient pools. In fact, in the cases where nutrient limitation was reported (Larned 1998, for tropical *C. sertularioides* and *C. racemosa*, and Terrados & Ros 1991, for Mediterranean *C. prolifera*), this was demonstrated in bioassays with detached plants, which were hence deprived of their sediment nutrient source; this was also the case in the first experiment reported here. Considering their low tissue N levels, plants in the second experiment were also N-limited. This limitation was most severe in the low-nitrogen treatments and can be held responsible for the difference in growth rates between the low- and high-nutrient treatments. These plants were grown on natural sediments; however, the vigorous washing with sulphuric acid and tap water prior to the experiment successfully removed organic material and nutrients. This is in agreement with the hypothesis put forward by Terrados & Ros (1991) that *C. prolifera* may be limited in sandy sediments that contain less organic material. Tissue N in *C. prolifera* from our field site varied between 2.8 and 4.0% DW, depending on season, and were always higher than tissue N levels in the seagrasses *Cymodocea nodosa* and *Zostera marina* collected from the same site at the same time (present paper and authors' unpubl. data). Considering the high critical level, *C. prolifera* at our sites was always slightly N-limited. In conclusion it can be said that, considering the low growth rate, high uptake capacity of the rhizoids and the capacity demonstrated in this paper to forage for nutrients, severe nutrient limitation is very unlikely to occur in *Caulerpa*, even in nutrient-poor waters, except on sediments containing little organic material.

Effects of irradiance and nitrogen levels on photosynthesis

Photosynthetic efficiency expressed as F_v/F_m was found to decrease under nutrient limitation stress in

many phytoplankton species (Geider et al. 1993, Yentsch et al. 2004). This was debated, however, by Parkhill et al. (2001), who found that F_v/F_m is rather insensitive to nutrient-limitation stress in phytoplankton cells in the steady state, experiencing balanced growth. Under these conditions the algae were able to acclimatise to chronically low nutrient availability and to maintain high photosynthetic efficiencies. This view was supported by the results of Young & Beardall (2003). Decreases in F_v/F_m as a result of nutrient limitation were also found in macroalgae (Henley et al. 1991, Korb & Gerard 2000, Gordillo et al. 2003); however, in all these studies algae were N-starved. The results from this study show that *Caulerpa prolifera* can maintain a high F_v/F_m under low, but constant, nutrient availability (nutrient limitation, Expt 1). Under nutrient starvation (Expt 2), a significant decrease in F_v/F_m was observed. Hence, we support the conclusion of Parkhill et al. (2001) that algae can maintain a high F_v/F_m under nutrient limitation (balanced growth), but not under nutrient starvation. The high variance in F_v/F_m between aquaria within treatment (significant nesting effect) and between assimilators within plants in the HL treatments impairs the interpretation of the results from the second experiment. Nevertheless, it can be clearly seen that nitrogen starvation leads to a reduction in maximum quantum yield.

The difference in q_N as shown in the RLCs found between plants cultivated at low and high light intensity clearly shows the capacity of *Caulerpa prolifera* of acclimatising to different light climates. This heat dissipation is considered a photoprotective mechanism (Ralph 1999, Bischof et al. 2002). The high I_k found in the HN treatments also indicates that *C. prolifera* is well adapted to high irradiances, provided that nitrogen is not limiting. This corroborates with the findings of Terrados & Ros (1992) and Gacia et al. (1996). Furthermore, the negative effect of nitrogen starvation is again demonstrated in the lower q_p values for the LN treatments. Despite the acclimatisation capacity of the photosystem to higher cultivation light levels, q_p , F_v/F_m and, ultimately, growth rates were highest in the HNLL treatment, indicating a preference for low or moderate growth irradiances. This supports the conclusion of Häder et al. (1997) that photoinhibition occurred in this alga even in its natural habitat when the sun was at high angles.

Concluding remarks

Caulerpa prolifera should be ecologically characterised as a slow-growing, nitrophilic species. Nitrogen limitation clearly stimulated stolon formation, indicating the capacity of the alga to forage for nutrients.

Further studies in which algae are cultivated on heterogeneous (with respect to nutrients) sediments, field inventories comparing algal morphology and sediment types and long-term experiments to study effects on the population are needed to check whether this morphological plasticity is indeed an adaptive feature.

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