Journal of Experimental Marine Biology and Ecology 407 (2011) 190–199

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Nitrogen uptake responses of *Gracilaria vermiculophylla* (Ohmi) Papenfuss under combined and single addition of nitrate and ammonium

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ARTICLE INFO

Article history:
Received 16 December 2010
Received in revised form 10 June 2011
Accepted 14 June 2011
Available online 23 July 2011

Keywords:
Ammonium
Ecophysiology
Gracilaria vermiculophylla
Nitrate
Nitrogen
Seaweeds

ABSTRACT

The ammonium (NH_4^+) and nitrate (NO_3^-) uptake responses of tetrasporophyte cultures from a Portuguese population of *Gracilaria vermiculophylla* were studied. Thalli were incubated at 5 nitrogen (N) levels, including single (50 μM of NH_4^+ or NO_3^-) and combined addition of each of the N sources. For the combined additions, the experimental conditions attempted to simulate 2 environments with high N availability (450 μM NO_3^- + 150 μM NH_4^+; 250 μM NO_3^- + 50 μM NH_4^+) and the mean N concentrations occurring at the estuarine environment of this population (30 μM NO_3^- + 5 μM NH_4^+). The uptake kinetics of NH_4^+ and NO_3^- were determined during a 4 h time-course experiment with N deprived algae. The experiment was continued up to 48 h, with media exchanges every 4 h. The uptake rates and efficiency of the two N sources were calculated for each time interval. For the first 4 h, *G. vermiculophylla* exhibited non-saturated uptake for both N sources even for the highest concentrations used. The uptake rates and efficiency calculated for that period ($V_{0-4\,h}$), respectively, increased and decreased with increasing substrate concentration. NO_3^- uptake rates were superior, ranging from 1.06 ± 0.1 to 9.65 ± 1.2 μM g(dw)$^{-1}$ h^{-1}, with efficiencies of 19% to 53%. NH_4^+ uptake rates were lower (0.32 ± 0.0 to 5.75 ± 0.08 μM g(dw)$^{-1}$ h^{-1}) but *G. vermiculophylla* removed 63% of the initial 150 μM and 100% at all other conditions. Uptake performance of both N sources decreased throughout the duration of the experiment and with N tissue accumulation. Both N sources were taken up during dark periods though with better results for NH_4^+. *Gracilaria vermiculophylla* was unable to take up NO_3^- at the highest concentration but compensated with a constant 27% NH_4^+ uptake through light and dark periods. N tissue accumulation was maximal at the highest N concentration (3.9 ± 0.25% dw) and superior under NH_4^+ (3.57 ± 0.2% dw) *vs* NO3 (3.06 ± 0.1% dw) enrichment. The successful proliferation of *G. vermiculophylla* in estuarine environments and its potential utilization as the biofilter component of Integrated Multi-Trophic Aquaculture (IMTA) are discussed.

1. Introduction

Nutrient availability is one of the key factors regulating the main physiological responses of seaweeds, with nitrogen being the most likely to limit their growth in temperate waters (DeBoer, 1981; Lobban and Harrison, 1997). In seawater, N is available to seaweeds in three major forms: nitrate (NO_3^-), ammonium (NH_4^+) and urea. The uptake rates of the different N sources can be affected by environmental parameters as well as by the seaweed species and their respective biology (Lobban and Harrison, 1997). Other factors known to influence N uptake are the nutritional history of the tissue (D'Elia and DeBoer, 1978; Fujita, 1985; Naldi and Wheeler, 2002), the nutrient concentration and its chemical species (DeBoer, 1981; Harrison and Hurd, 2001), and even genetics (Lobban and Harrison, 1997).

The co-occurrence of the different chemical N forms can have an antagonistic effect on the uptake. NH_4^+concentrations as low as 5 μM have been found to inhibit or even suppress the uptake of NO_3^- by some seaweed species (D'Elia and DeBoer, 1978; Haines and Wheeler, 1978; Smit, 2002; Thomas and Harrison, 1987). At the same time, most seaweeds have a higher affinity for the NH_4^+- N source (DeBoer et al., 1978; D'Elia and DeBoer, 1978; Hanisak, 1983; Lobban and Harrison, 1997; Naldi and Wheeler, 1999; Pereira et al., 2008; Phillips and Hurd, 2003, 2004; Smit, 2002), probably due to the low levels of energy required to assimilate this nutrient (DeBoer, 1981; Lobban and Harrison, 1997). In fact, NH_4^+ uptake often occurs by passive diffusion, meaning that the uptake rate increases proportionally to the substrate concentration. Nitrate uptake, on the other hand, typically shows saturation kinetics, meaning that with increasing substrate concentrations, the uptake capacity reaches a maximum. In this case, the N

Abbreviations: IMTA, Integrated Multi-Trophic Aquaculture.

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doi:10.1016/j.jembe.2011.06.034

uptake is an energy-dependent process and can be described by the Michaelis–Menten equation: $V = V_{max} \times S / K_s + S$ (DeBoer, 1981).

Gracilaria vermiculophylla thrives in Ria de Aveiro (40° 38′N; 8° 43′W). This geographical area suffers from strong anthropogenic influence, such as cities, tourism, boat activities, fisheries, earthen pond aquaculture and runoffs from agriculture. All these aspects turn this lagoon into a site with high nitrogen concentrations (essentially nitrates), leading to eutrophication (Borrego, 1993; Lopes et al., 2007; Silva et al., 2002). The non-native seaweed *G. vermiculophylla* is currently the dominant species in an intertidal flora community also composed by *Zostera noltii*, *Ulva* spp. and *Fucus* sp. (Abreu et al., submitted for publication; Silva et al., 2004). The rapid expansion of non-native species can have negative impacts on the abundance of slow growing communities, such as seagrasses (Occhipinti-Ambrogi, 2007; Williams, 2007). The ability to take up and store nutrients is one of the factors affecting the dominance of species in intertidal communities. Fast growing marine algae have higher nitrogen requirements than slow growing perennial species such as *Fucus* sp. and other benthic macrophytes, like seagrasses. Thus, fast growing species are positively affected by increased nutrient availability (Pedersen and Borum, 1996; Rosenberg and Ramus, 1982).

Environmentally friendly aquaculture practices like Integrated Multi-Trophic Aquaculture (IMTA), where fed species (*e.g.* fish, shellfish) are cultivated together with nutrient extractive species (seaweeds), are now widely supported (see review by Chopin et al., 2008). Fed aquaculture can be implemented in an intensive (100% depended of manufactured feeds) or semi-intensive (natural + commercial food supply) manner. These systems differ in several features, one of them being the N load in their discharge waters. Fish effluents include mainly NH_4^+ or NO_3^-, but often both, depending on the type of aquaculture and on the existence of any installed biofiltration process. In general, the nitrate fraction is higher in the outflow of semi-intensive systems and in intensive fish farms with water recirculation practices. On the other hand, intensive aquaculture companies without recirculation systems will discharge an effluent highly enriched with ammonium (Neori et al., 2004). Some *Gracilaria* species have proven to be efficient biofilters in IMTA systems (Abreu et al., 2009; Buschmann et al., 2008; Troell et al., 1997), carrying out an important environmental service for the aquaculture industry. Fish aquaculture is the main activity in the earthen ponds of Ria de Aveiro lagoon (semi-intensive systems) and also in surrounding land-based intensive systems. The integration of *G. vermiculophylla* as the biofilter component of these systems might be a good solution to reduce the N loads from these activities into the recipient environment.

A profound knowledge of the N uptake response of *G. vermiculophylla* is important essentially for two reasons. It can help to explain the position of the species in the intertidal community of the Ria de Aveiro ecosystem and, on the other hand, is fundamental to assess its potential as a biofilter in IMTA systems. This work intended to provide more information regarding the N dynamics of *G. vermiculophylla*. Through a short-term experiment, the kinetics of NH_4^+ and NO_3^- uptake were described. During a 48 h period, the uptake efficiency of these N sources was studied in order to determine diel uptake differences and thus estimate the overall biofiltration efficiency of *G. vermiculophylla*. The influence of the N source and concentration on the growth, N uptake and tissue accumulation of *G. vermiculophylla* are discussed.

2. Methodology

2.1. Preliminary experiment

To determine the optimal stocking density for the N uptake experiments, we evaluated the Relative Growth Rates (RGR), yield and daily pH variation of *G. vermiculophylla* set at 0.5, 1.0, 2.0 and 4.0 g(fw) l^{-1}. Tetrasporophyte thalli of *Gracilaria*, from different strains obtained in the lab (Abreu et al., submitted for publication), were incubated in 1 l Erlenmeyer flasks containing 800 ml of autoclaved sea water (ASW) enriched with Von Stosch's medium (VSE) (Ott, 1965). All glass material used during the experiment had been acid washed with 10% HCl to prevent possible nutrient contamination. Four replicates were used for each experimental condition. Biomass from the different strains was randomly assigned to each replicate. The experiment was carried out inside a walk-in culture chamber (Harris Environmental Systems, Maine, USA) at 20 °C, with constant photon flux density (150 μmol photons m^{-2} s^{-1}) and photoperiod (12:12; Light:Dark). These conditions were found to be non-limiting for growth of *G. vermiculophylla* (Abreu et al., submitted for publication). The seaweeds were kept in constant movement through bottom aeration in the flask.

The experiment lasted 14 days. Every 3–4 days, the *Gracilaria* thalli were blotted dry and the fresh weight determined. At this time, the biomass was trimmed to the initial stocking density and the culture media was replaced. The RGR for the total duration of the experiment was an average of the RGR observed for each 3–4 day interval. These were calculated as:

$$RGR\left(\%\ day^{-1}\right) = \frac{[Ln\ (\mathrm{FW}) - Ln\ (\mathrm{IW})]}{\mathrm{T}*100}$$

where FW = Final fresh weight, IW = Initial fresh weight and T = Days in culture (3 or 4). In the same way, the yield of each experimental condition was calculated as an average of the biomass increase registered at each time interval.

In the morning of the last period of the experiment, after restocking the flasks with the correct biomass, the pH was monitored after 1, 3, 6, 18, 20 and 24 h. At each time, 2 replicates from each treatment were monitored with a pH sensor (Denver Instrument, NY, USA).

2.2. Seaweed pre-treatment and experimental conditions

The experimental work was carried out with a tetrasporophyte strain which was obtained from laboratory reared *G. vermiculophylla* carposporophytic material originally from Ria de Aveiro lagoon, Portugal (40°38″N, 8°43″W). One week before the experiment, the seaweeds were incubated in aged ASW, enriched with modified VSE (no N or P added) to lower the N content in the tissue of *G. vermiculophylla*. During this time and throughout the experiment, all the cultures were kept at the same conditions of temperature, photon flux density and photoperiod used in the preliminary experiment.

The time-course N uptake experiments followed the mixed model (perturbation + multiple flasks) as described by Pedersen (1994) and involved the addition, to N-deprived ASW, of multiple and single N-sources (Table 1). The multiple N-addition conditions were chosen in order to mimic environments of high nitrogen availability at different levels. Treatment A represents the effluent of intensive land-based aquaculture, with extreme values of NH_4^+ and NO_3^- (based on values from a commercial fish farm in Portugal); B stands for aquaculture environments with some effluent treatment and for semi-intensive systems; C represents the average nitrogen concentrations at Ria de Aveiro lagoon (Lopes et al., 2007). The single N source treatments intended to compare the nitrogen uptake performance of *G. vermiculophylla* when in the presence of NH_4^+ or NO_3^-; the concentration of 50 μM for each nutrient

Table 1
Nutrient concentrations (μM) used to set up the experimental culture media consisting of 5 levels of N enrichment with combined (A, B and C) and single (D, E) NH_4^+ or NO_3^- sources. P was added to maintain the N:P ratio equal to 10.

Experimental treatment	NH_4^+ (μM) (NH_4Cl)	NO_3^- (μM) ($NaNO_3$)	PO_4^{3-} (μM) ($Na_2HPO_4.12H_2O$)
A	150	450	60
B	50	250	30
C	5	30	3.5
D	50	0	5.0
E	0	50	5.0

(Table 1) was expected to be non-limiting for the duration of the experiment. The molar N:P ratio was kept at 10:1. Four replicates + two controls (no algal material added) were established per treatment.

NH_4Cl and $NaNO_3$ stock solutions were prepared daily to guarantee the correct concentration for each experimental treatment. The $Na_2HPO_4^{2-}.12H_2O$ stock solution, due to its low volatility, was prepared in enough quantity to cover the needs of the entire experiment. An initial stocking density of 2 g (fw) l^{-1} was used for the N uptake experiments, as determined by the preliminary experiment. Seaweeds were incubated in 1 l acid washed Erlenmeyer flasks containing 800 ml of the respective experimental media. Water motion was assured by constant aeration to the flasks.

2.3. Short-term (4 h) N uptake experiment

This experiment was initiated immediately after lights went on inside the culture chamber. The nutrient depletion of the medium was determined by nutrient analysis of water samples. In order to determine the initial N concentrations, 10 ml water samples were taken before adding the seaweed. Further 10 ml water samples were collected at each time interval: 15, 30, 45, 60, 90, 180 and 240 min.

2.4. Medium-term (2 day) N uptake experiment

This experiment was the follow up of the short-term one (Section 2.3). In this case, 10 ml water samples were collected at 0, 4, 8, 12, 16 and 24 h for all experimental treatments. At these times, the biomass inside the flask was weighed and, if necessary, trimmed back to the experimental stocking density, (2.0 g (fw) l^{-1}).The total incubation volume of the flasks was replaced at every sampling moment, except for the sampling occurring in the dark (+16 h). For every medium change, an initial water sample was collected before adding the algae. Water samples at +12 and +24 h were taken before lights inside the culture chamber went off or on, respectively. This procedure was repeated for 2 consecutive days, with the same *G. vermiculophylla* biomass.

Water samples from both experiments were kept at −20 °C, until analysis (less than a month after). Analysis of NH_4^+, NO_3^- and PO_4^{3+} was carried out using a Four-Channel Auto Analyzer equipped with High-Sensitivity Seawater Cartridges (Lachat-QuikChem AE Ion Analyzer; Hach, Loveland, CO) at the Avery Point Campus of the University of Connecticut.

Algal material was dried to a constant weight (48 h) in an oven kept at 60 °C, for determination of dry weight (dw). Carbon and nitrogen tissue content (% dw) was determined using a CHNS/O Analyser (Series II, 2400 Perkin-Elmer).

2.5. Trends in Nitrogen tissue content of wild Gracilaria vermiculophylla

Samples (n = 4) from a *G. vermiculophylla* population thriving in Ria de Aveiro lagoon were collected between February 2007 and July 2009 and later analyzed for nitrogen tissue content (% dw), following the procedure previously described in Section 2.4.

2.6. Calculations

2.6.1. N uptake rates and kinetic parameters

For the short (4 h) and medium-term (48 h) experiments, uptake rates for each N source during each time interval were calculated using the equation:

$$V = \frac{(Si–Sf) \times vol}{t \times dw}$$

where V = uptake rate (μmol g(dw)$^{-1}$ h^{-1}), Si = substrate concentration at start of time interval (μM), Sf = substrate concentration at end of time interval (μM), vol = volume at start of time interval (l), t = time (h), and dw = dry weight (g).

The data from time intervals with suppressed or enhanced uptake (observed from the depletion curves), where not considered for uptake rates determination (see Pedersen and Borum, 1997). For estimation of the kinetic parameters of each N source, uptake rates from the 5 treatments were plotted together in V *vs* S curves. The V (uptake rate) against V/S ($S = NH_4^+$ or NO_3^- concentration) linear transformation of the Michaelis–Menten equation was used to determine whether the data could be described by Michalies–Menten kinetics (Dowd and Riggs, 1965). Negative values and data points that appeared erroneous (outliers) were excluded from the analysis. The uptake efficiency of *Gracilaria* for each nutrient (percentage of the nutrient removed from the media) was determined using the equation $[(S_i - S_f)/S_i \times 100]$.

2.6.2. Growth rate

The growth rate of the algae under the different experimental conditions was determined after 48 h and, when time permitted, for the time interval between the culture medium exchanges. Calculation was based in the same formula used for the preliminary experiment (Section 2.1) but with RGR expressed as% h^{-1} (T = number of hours in culture).

2.7. Data analysis

ANOVA was used to analyze the responses of *G. vermiculophylla* to the different N enrichment conditions in terms of uptake rate and efficiency of each nutrient (NH_4^+ and NO_3^-), growth rates, C and N tissue content and C:N ratio. In the case of the short experiment, ANOVA was applied directly to the uptake variables calculated between initial (0 h) and final sampling times (4 h). However, for the 48 h experiment, and to avoid time dependency of the data, the ANOVA was applied for the calculated mean of the uptake variables determined for each time interval. This same procedure was done to determine differences in the nutrient uptake variables between light and dark periods.

Cochran's heterogeneity of variances test was applied to the data and when the result was significant, data were transformed. If data normalization was not achieved, a more conservative level of significance ($p<0.01$) was applied (Underwood, 1997). Significant differences between treatments were analyzed *a posteriori* with a Student–Newman Keuls (SNK) test ($\alpha = 0.05$). All statistical analyses were performed using the software GMAV v5 (EICC, The University of Sydney, Australia) licensed to Maria H. Abreu.

3. Results

3.1. Preliminary experiment

G. vermiculophylla testrasporophytes had the best overall growth performance (best relation between RGR and yield) when cultured at 2 g(fw) l^{-1} (Fig.1-B). RGR was close to 8% day^{-1}, translated into a biomass production near 0.4 g(fw) day^{-1}. As seen in Fig. 1-A, the pH values ranged between 7.8 and 8.9, with lower values registered after dark periods (+18 h). In order to prevent high levels of ammonium volatilization, it is important to maintain pH levels close to 8 (Hargreaves, 1998). After 6 h in culture, all treatments were already above that value. As expected, the increase in pH levels was proportional to increasing stocking densities.

Combining the two sets of information, it was decided that a stocking density of 2 g(fw) l^{-1}, would be appropriate for the nitrogen uptake experiment.

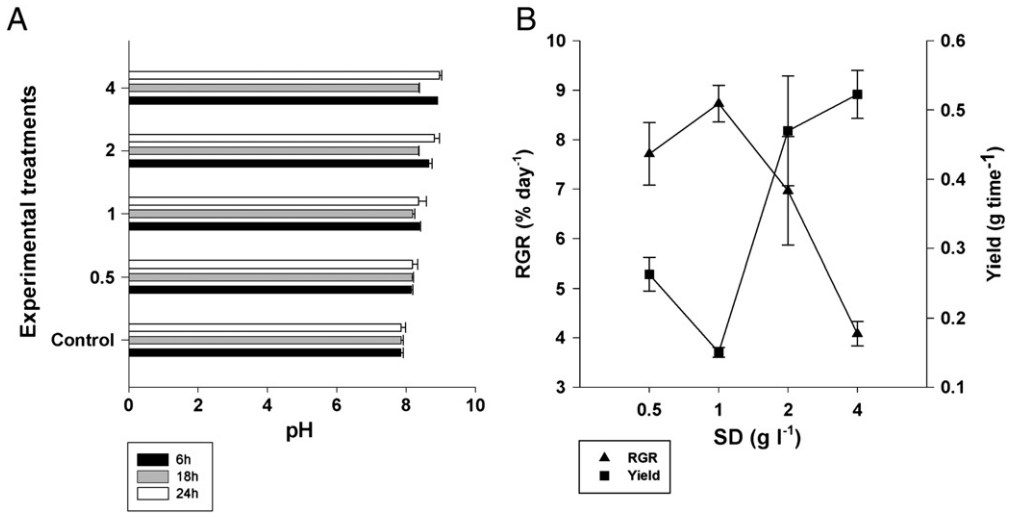

Fig. 1. Results from the preliminary experiment testing different stocking densities (0.5, 1.0, 2.0 and 4.0 g l^{-1}) A — Variation of pH values in the flasks stocked at different densities and a control with no seaweed added after 6, 18 and 24 h in culture (mean ± SE, n = 2). B — Growth rates and yield (mean ± SE, n = 4).

3.2. NH_4^+ and NO_3^- uptake kinetics

The change in NH_4^+ or NO_3^- concentration in control flasks (no algae) was residual; therefore the depletion of NH_4^+ and NO_3^- in treatment flasks was attributed to uptake by the tetrasporophytes of *G. vermiculophylla*. Small deviations in the real concentrations compared to the planned ones (essentially for NO_3^-) are visible in some treatments, but none should have influenced the observed results.

G. vermiculophylla removed both NH_4^+ and NO_3^- at all the conditions tested (Fig. 2). NH_4^+ was totally depleted in nearly all treatments. At the highest N concentrations (A & B), NH_4^+ depletion was suppressed during the first 30 min. Therefore, the uptake rates were not calculated for those time intervals. The V *vs* V/S curve for this nutrient did not point to saturation kinetics. In fact, NH_4^+ uptake rate was linear, increasing with increasing substrate concentration (Fig.3, $r^2 = 0.77$). On the other hand, NO_3^- depletion was never complete (even when it was the only N source) and was also erratic, especially in the highest concentrations (Fig. 2, treatments A and B). In all treatments, except for B, NO_3^- concentration increased in the culture medium during the first 15 and even 30 min.

In treatments A and B a faster nitrate depletion was coincident with the NH_4^+ uptake suppression (Fig. 2); however after 60 min nitrate depletion was very slow, almost inexistent. It was possible to distinguish a different V–S relation between the highest and lowest

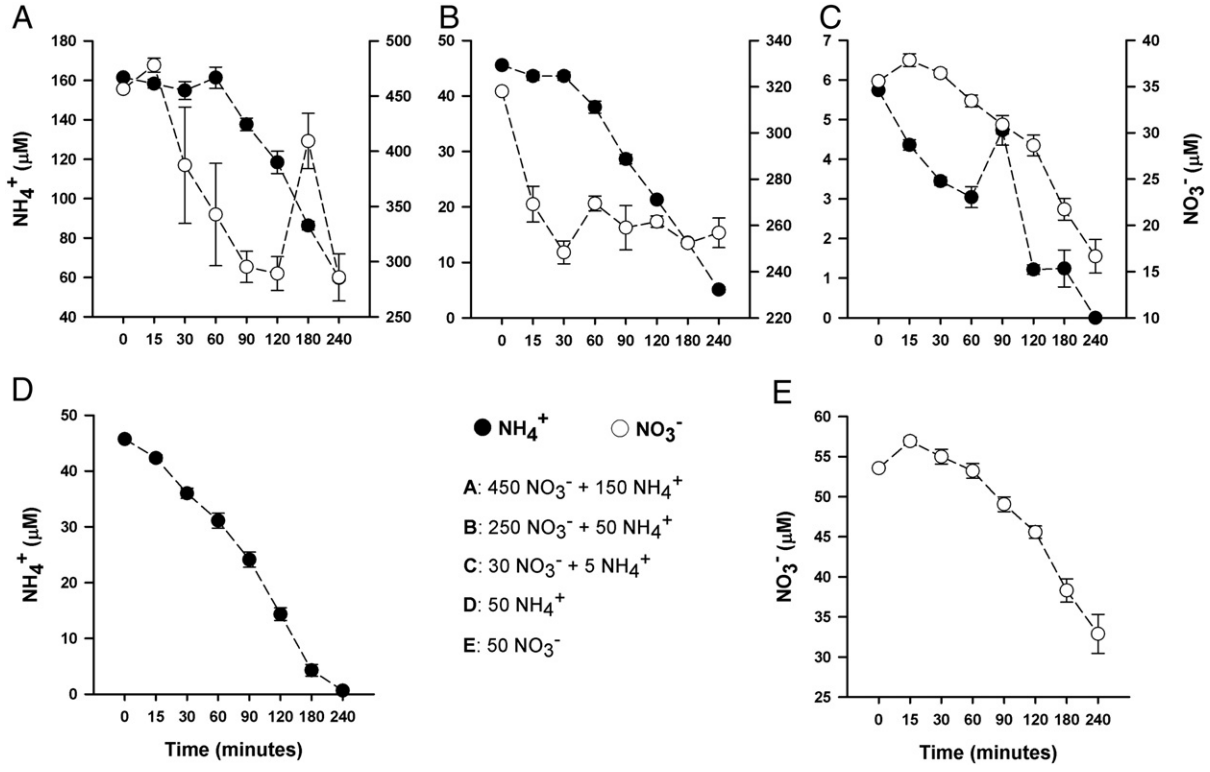

Fig. 2. Time course of NH_4^+ (black circles) and NO_3^- (open circles) depletion by tetrasporophytes of *Gracilaria vermiculophylla* cultured in media enriched with both N sources (A, B and C) and with only ammonia (D) or nitrate (E). Symbols represent mean ± SE, n = 4. The time intervals with spikes in N concentration were not considered for uptake rates calculations.

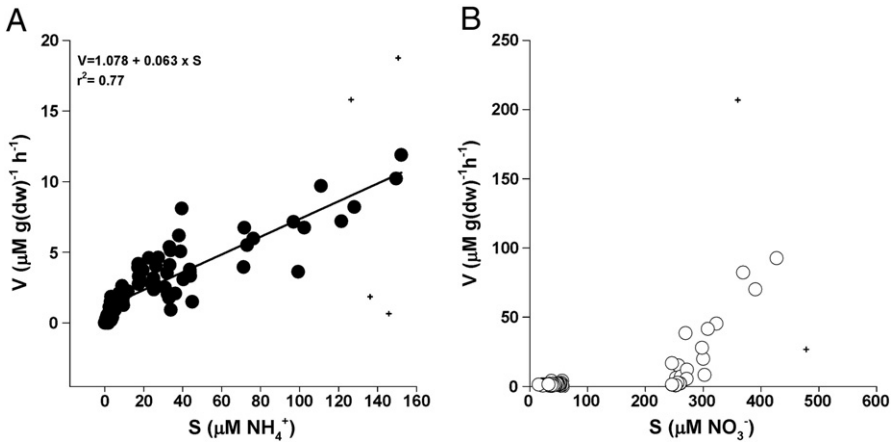

Fig. 3. Rate of NH_4^+ (A) and NO_3^- (B) uptake (V) as a function of substrate concentration (S) by *Gracilaria vermiculophylla* tetrasporophytes. Linear regressions are fitted to pooled data from the 4 h time-course experiment. Symbols represent replicates, except cross-hairs (+), which indicate outliers omitted from regression analyses.

NO_3^- concentrations tested (Fig.3). At the highest concentration, V–S was linearly positive; on the other hand, for the lowest NO_3^- concentrations (up to ca. 60 µM), V–S was non-linear with slower uptake values (0.3 to 2.6 µM g(dw)$^{-1}$ h^{-1}). Similarly to the NH_4^+, the V *vs* V/S linear transformation of the Michaelis–Menten did not reveal saturation kinetics for NO_3^- uptake, even when we tried to apply it to differentiated concentration intervals (0–60 µM and 200–400 µM).

3.3. NH_4^+ and NO_3^- uptake (rates & efficiency)

G. vermiculophylla had an overall positive uptake of both NH_4^+ and NO_3^-. For both nutrients, uptake rates ($V_{0\text{-}4h}$) increased with N enrichment levels, as previously seen by uptake kinetics patterns, while uptake efficiency decreased (Figs. 4 and 5). Under the presence of both N sources (Fig. 4-A), *G. vermiculophylla* had the highest uptake rates for NO_3^-, while in single addition conditions the NH_4^+ uptake was higher (2.52 ± 0.02 µM NH_4^+ g(dw)$^{-1}$ h^{-1} *vs* 1.17 ± 0.14 µM NO_3^- g(dw)$^{-1}$ h^{-1}). Nitrate uptake rates were significantly higher under treatment A (9.6 ± 1.2 µM g(dw)$^{-1}$ h^{-1}; $F_{(3,15)} = 39.99$, $P<0.05$; Fig. 4-A) with no differences observed for the other conditions. In the case of NH_4^+, uptake rates were also significantly affected by the experimental conditions ($F_{(3,15)} = 3193.4$, $P<0.05$; Fig. 4-A). Maximum uptake rates of NH_4^+ (5.7 ± 0.07 µM g(dw)$^{-1}$ h^{-1}) occurred at the highest concentration. For the same substrate concentration (50 µM, treatments B and D), *Gracilaria* removed NH_4^+ at equal rates, independently of the concentration of NO_3^- (treatment B). The lowest NH_4^+ uptake rate (0.32 ± 0.00 µM g(dw)$^{-1}$ h^{-1}) was recorded under the lowest NH_4^+ concentration of 5 µM (treatment C).

In terms of the NH_4^+ uptake efficiency by *G. vermiculophylla*, which accounts for the initial substrate concentration, the results clearly show that this was always the preferred N source. Values of uptake efficiency were ca. 100% (Fig. 4-B), except under treatment A ($F_{(3,15)} = 773.45$, $P<0.05$; Fig.4-B). Even so, *G. vermiculophylla* removed 63% of the initial ca. 150 µM NH_4^+. Regarding the NO_3^- uptake efficiency, it was similar for treatments A, C and E with values between 33 and 58%. A significantly lower nitrate uptake value (19.2 ± 2.01%) was observed for *Gracilaria* subjected to treatment B ($F_{(3,15)} = 10.68$, $P<0.05$).

As the experiment progressed with time (48 h), the mean values of NH_4^+ and NO_3^- uptake rates and efficiency decreased (Fig. 5). The two variables were significantly influenced by the experimental conditions, as shown by the results of the ANOVA for NH_4^+ ($F_{(3,15)} = 934.71$, $P<0.05$, Fig. 5-A; $F_{(3,15)} = 441.99$, $P<0.05$, Fig. 5-B) and for NO_3^- ($F_{(3,15)} = 103.83$, $P<0.05$, Fig. 5-A; $F_{(3,15)} = 112.76$, $P<0.05$, Fig. 5-B). The efficiency in NH_4^+ uptake kept the pattern observed during the short-term experiment, with maximal efficiency at the lowest concentration (95.4 ± 0.8%) and minimal at the highest (27.0 ± 0.8%). NO_3^- uptake, on the other hand, seems to have been much more affected by the continuous pulses of N enrichment (every 4 h). In fact, *Gracilaria* was unable to remove NO_3^- under the highest N enrichment condition. Furthermore, the highest uptake efficiency (55.0 ± 3.9%, Fig. 5-B) was observed when the algae were subjected to the lowest NO_3^- concentration. The inability of *G. vermiculophylla* to cope with the high nitrate concentrations at treatment A is clear in Fig. 6. For the combined N source treatments, *G. vermiculophylla* took up more nitrogen as nitrate than ammonia, except under treatment A.

When comparing the diel uptake performance, the uptake during the dark periods was lower for all treatments (Fig. 7) but maintained the same general differences between them (Fig. 8). Under treatments

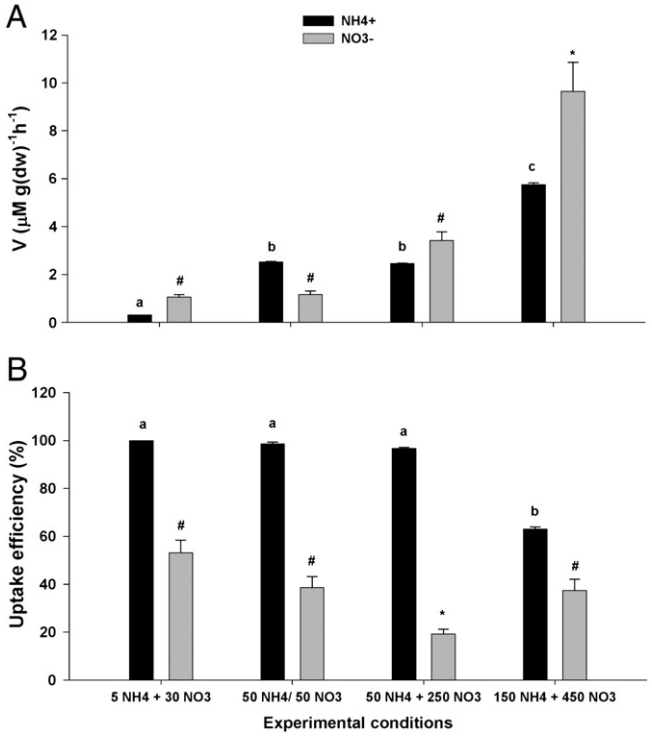

Fig. 4. Uptake rates (A) and uptake efficiency (B) of NH_4^+ and NO_3^- by tetrasporophytes of *Gracilaria vermiculophylla* calculated for the entire short-term experiment (0–4 h). Mean ± S.E., n = 4. Different letters (NH_4^+) or symbols (NO_3^-) above bars indicate significant differences between the experimental conditions ($p<0.05$).

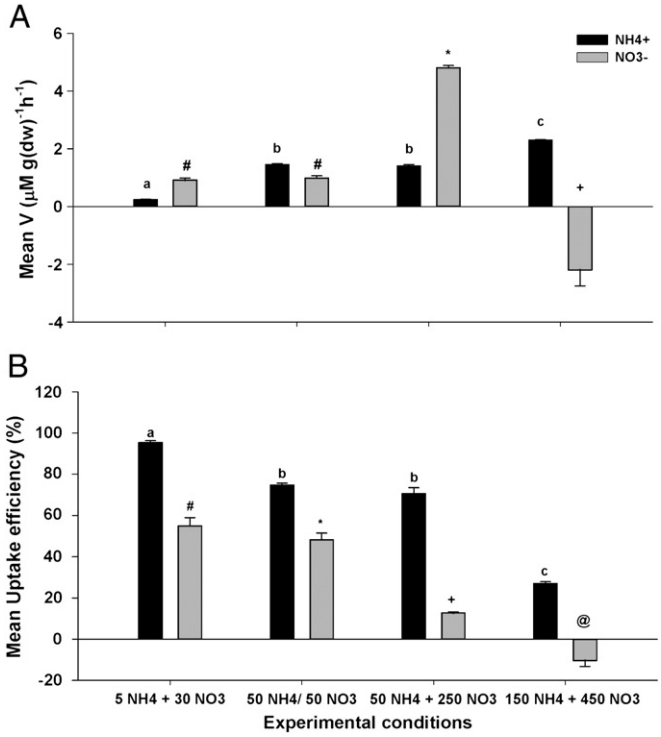

Fig. 5. Uptake rates (A) and uptake efficiency (B) of NH_4^+ and NO_3^- by tetrasporophytes of *Gracilaria vermiculophylla* over a 48 h period with 4 daily exchanges of the experimental culture media. Mean ± S.E., n = 4. Different letters (NH_4^+) or symbols (NO_3^-) above bars indicate significant differences between the experimental conditions ($p<0.05$).

C (5 μM), B and D (50 μM), *Gracilaria* was able to remove all the NH_4^+ present in the water after every nutrient pulse. Thus, during the night period with no media replacement (10 pm to 6 am), uptake rate values were low (Fig.7) but with mean uptake efficiencies ranging from 61.8 ± 2.6% to 81.7 ± 3.3% (Fig.8-A). In treatment C, with the lowest total N concentration (35 μM), the nitrogen (NH_4^+ + NO_3^-) was depleted up to residual values. In treatment B, during dark periods, the high removal of NH_4^+ was coincident with an increase in the nitrate uptake efficiency by *Gracilaria* (Fig. 8). Under treatment A, with highest N enrichment levels, *Gracilaria* removed ca. 27% of the

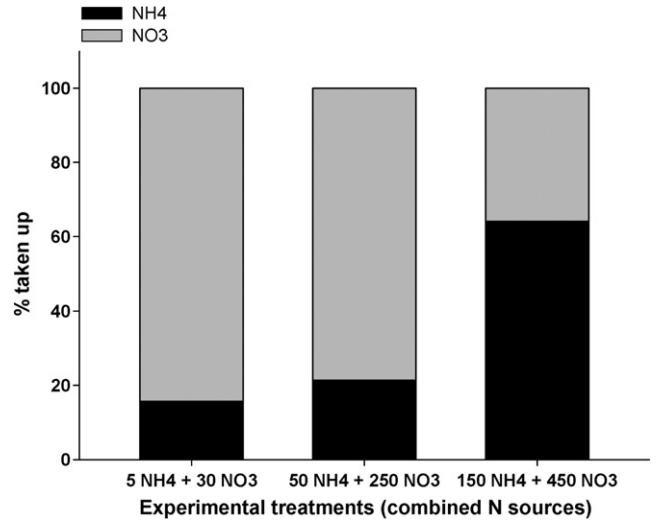

Fig. 6. Percentage of NH_4^+ and NO_3 of the total N taken up by *Gracilaria vermiculophylla* after 48 h in the combined source treatments. Mean values, n = 4.

NH_4^+ for both light and dark periods (Fig. 8), but was unable to uptake NO_3^-.

3.4. Ammonium and nitrate uptake efficiency vs nutrient tissue content

The analysis of total carbon and nitrogen content in the tissue of *G. vermiculophylla* revealed a significant effect of the experimental conditions but only for the accumulation of nitrogen ($F_{(4,19)} = 5.86$, $P<0.05$; Fig. 9).

Gracilaria from treatments C (lowest N concentration — 35 μM) and E (only nitrate added — 50 μM), had the lowest N content (3.06 ± 0.10% dw). The accumulation of N for the other conditions was similar and with a maximal value of 3.92 ± 0.25% dw at the highest N enrichment level (A). Considering this pattern of N accumulation, the uptake efficiency of both N sources decreased with an increasing N content, as clearly shown in Fig. 10. C:N ratio was also affected by the experimental treatments ($F_{(4,19)} = 5.56$, $P<0.05$). Values ranged from 7.58 ± 0.17 to 9.23 ± 0.36 and were attained at the highest and lowest N substrate concentrations, respectively. It is worth notice that, in the presence of only NH_4^+ or NO_3^- (treatments D and E), *G. vermiculophylla* accumulated more N in its tissue with NH_4^+.

The growth rate of *G. vermiculophylla* tetrasporophytes was of 0.69 ± 0.02% h^{-1} and was not influenced by the experimental conditions ($P>0.05$). Nonetheless, *G. vermiculophylla* RGR was slightly higher with NH_4^+ and decreased with decreasing nutrient concentrations.

3.5. Nitrogen tissue content in Gracilaria vermiculophylla wild biomass

The nitrogen accumulation in the tissue of *G. vermiculophylla* thriving in Ria de Aveiro lagoon exhibited a clear seasonal pattern (Fig. 11). Values above 5% N (DW) were registered in the colder months and the lowest N accumulation values (ca. 3% DW) were observed in spring and summer months.

4. Discussion

This work showed that *G. vermiculophylla* can assimilate nitrogen even at concentrations as high as 600 μM. With increasing nitrogen concentration, the nitrogen uptake rate and nitrogen tissue content increased but the uptake efficiency decreased. Generally, the uptake performance of NH_4^+ and NO_3^- by *G. vermiculophylla* decreased with time and N tissue accumulation. *G. vermiculophylla* was more efficient in taking up NH_4^+, also during dark periods.

The uptake of NH_4^+ by *G. vermiculophylla* is carried out by simple diffusion as revealed by the non-saturated uptake kinetics observed in this study. This same kind of kinetics has been described for other *Gracilaria* species: *Gracilaria tikvahiae* (Friedlander and Dawes, 1985), *Gracilaria pacifica* (Naldi and Wheeler, 2002) and *Gracilaria gracilis* (Smit, 2002). The observed enhanced NH_4^+ uptake by *G. vermiculophylla* in the lower concentrations is common to other N deprived seaweed (Pedersen and Borum, 1997), including *Gracilaria* species (D'Elia and DeBoer, 1978; Thomas and Harrison, 1987).

Normally, NO_3^- uptake shows saturation kinetics (DeBoer, 1981; Lobban and Harrison, 1997). In our case, during the short term experiment and similarly to NH_4^+, NO_3^- uptake increased with increasing external NO_3^- concentration. This linear relation was already reported for *G. pacifica* (Thomas et al., 1987). However, after the observed enhanced NO_3^- uptake and a lag phase in NH_4^+ uptake during the first 30 to 60 min, the depletion of NH_4^+ was faster, with *G. vermiculophylla* taking up 80 to 100% of this nutrient's initial concentration. We could not find any literature reporting this phenomenon. The normal pattern is that N deprived algae, when exposed to pulses of inorganic N, exhibit a slower NO_3^- uptake and enhanced NH_4^+ uptake (Harrison et al., 1989; Kraemer et al., 2004; McGlathery et al., 1996; Pedersen and Borum, 1997; Smit, 2002). However, none of these studies worked with combined NH_4^+ and NO_3^- additions in

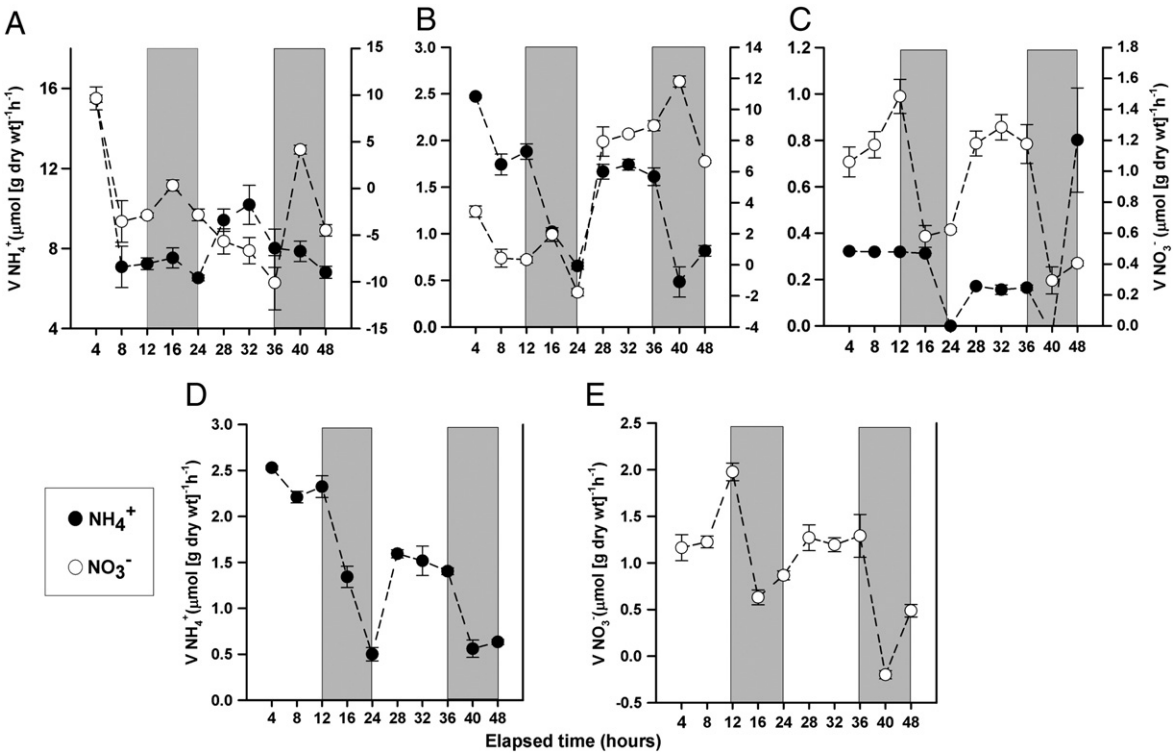

Fig. 7. Mean (± SE, n = 4) diel changes in the uptake rates of NH_4^+ and NO_3^- by the tetrasporophytes of *Gracilaria vermiculophylla* during a 48 h period with 4 daily exchanges of the experimental culture media. Observations made for 5 N enrichment levels (Table 1) with combined (A, B, C) and single addition of NH_4^+ (D) or NO_3^- (E). Shaded area = period of darkness.

such high concentrations as the ones used in this present study. Passive diffusion of NO_3^- may be significant at substrate concentrations above environmental concentrations (Lobban and Harrison, 1997). One possible explanation for this observation, is that the external NO_3^- concentrations were so high (250 and 450 µM) that *Gracilaria* filled the intracellular N pools with this N source. Consequently, NO_3^- uptake probably became suppressed (Fujita, 1985; Naldi and Wheeler, 2002) and the seaweed initiated the N storage by NH_4^+ passive diffusion (beneficial in terms of energy expense). Unlike other *Gracilaria* species (*G. pacifica* — Thomas and Harrison, 1987; *G. gracilis* — D'Elia and DeBoer, 1978; Smit, 2002), *G. vermiculophylla* had similar NO_3^- uptake rates in single and combined treatments with equivalent $NO3^-$ concentration (C and E), with no apparent inhibition due to the presence of NH_4^+. The inhibitory effect of ammonium over nitrate uptake is widely accepted, however, Dortch (1990) working with phytoplankton, described it as a highly variable phenomenon.

G. vermiculophylla was more efficient in depleting NH_4^+ than NO_3^-. When in N deprived conditions, it was able to uptake 106 µmoles of NH_4^+ in 4 h. High levels of NH_4^+ uptake were also reported by Naldi and Wheeler (2002) for *G. pacifica* (500 µmoles in 24 h). *G. tikvahiae*

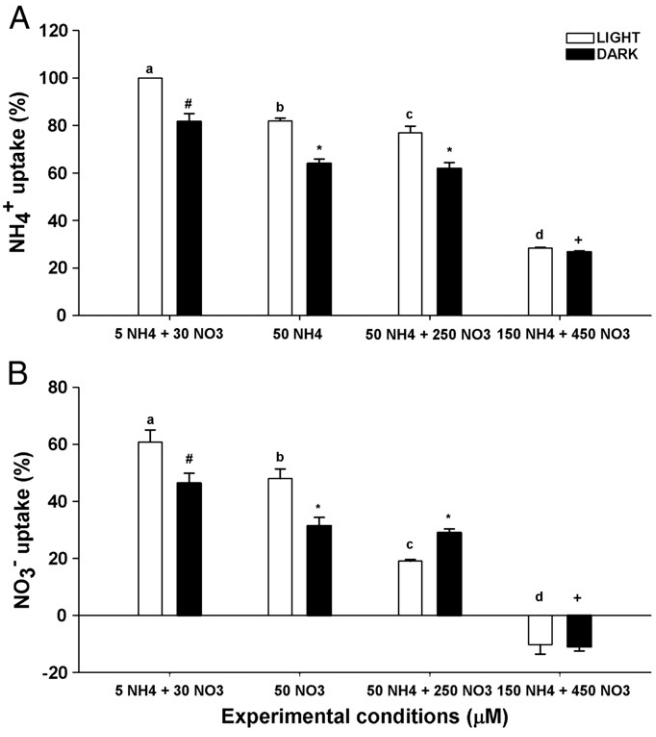

Fig. 8. Uptake efficiency during dark and light periods of NH_4^+ (A) and NO_3^- (B) by tetrasporophytes of *Gracilaria vermiculophylla* cultured for 48 h under combined and single nitrogen enrichment treatments. Mean ± S.E., n = 4. Different letters (light) or symbols (dark) above bars indicate significant differences between the experimental conditions ($p<0.05$).

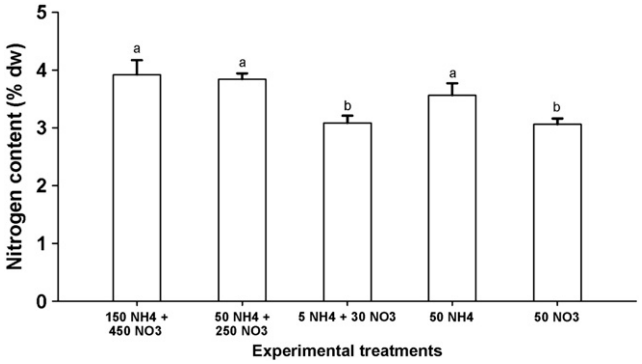

Fig. 9. Nitrogen tissue content of *Gracilaria vermiculophylla* tetrasporophytes cultured for 48 h under the 5 N enrichment experimental treatments, with 4 daily culture media exchanges. Mean ± S.E., n = 4. Different letters above bars indicate significant differences between treatments ($p<0.05$).

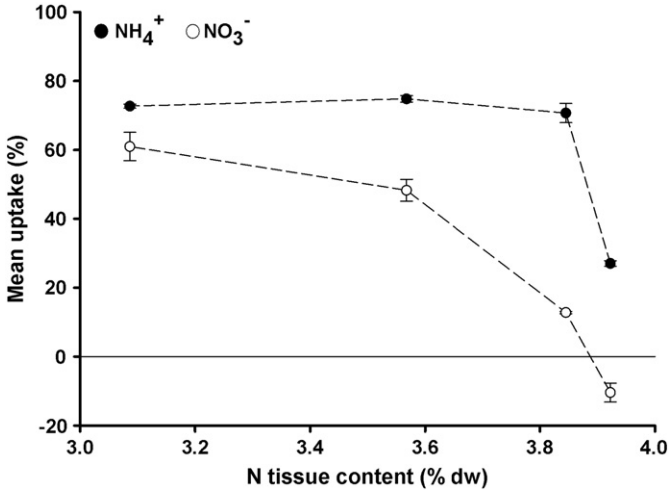

Fig. 10. Uptake efficiency of NH_4^+ and NO_3^- by tetrasporophytes of *Gracilaria vermiculophylla* as a function of their nitrogen tissue content 48 h after the start of the experiment. Symbols represent mean (± SE, n = 4) uptake efficiency values for each experimental treatment (see Table 1).

and *G. gracilis* (D'Elia and DeBoer, 1978; Ryther et al., 1981; Smit, 2002) as well as several other algae species (Phillips and Hurd, 2003; Pereira et al., 2008;) have also shown to be more effective in removing NH_4^+ when in presence of combined N sources.

A decrease in the overall uptake performance of NH_4^+ and NO_3^- by *G. vermiculophylla* was observed after 48 h of experiment. A similar decrease in N uptake rates with time of exposure to nutrients was previously observed in other studies (Lartigue and Sherman, 2005; Naldi and Wheeler, 2002; Pedersen et al., 2004).The total filling of the intracellular N pool probably explains this phenomenon. Our results showed that uptake efficiency of both N sources, but especially NO_3^-, decreased with increasing nitrogen tissue accumulation. Several studies have shown that seaweeds accumulate N in their tissue as a function of the dissolved nitrogen levels present in the water (*Gracilaria* spp. — Bird et al., 1982; Horrocks et al., 1995; Rosenberg and Ramus, 1982; *Ulva rigida* — Naldi and Viaroli, 2002; *Palmaria palmata* — Martinez and Rico, 2002). Studies with ^{15}N labeling reported that same pattern for nitrogen uptake by *G. vermiculophylla* (Tyler and McGlathery, 2006). In the highest concentration tested (treatment A), for which *G. vermiculophylla* had the highest N tissue

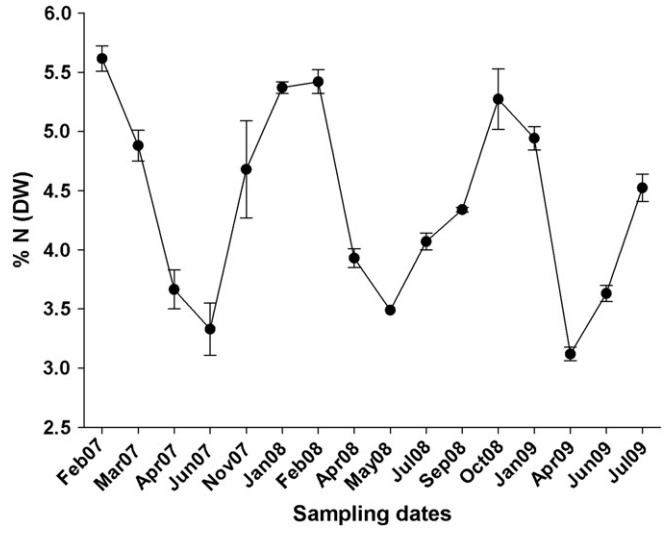

Fig. 11. Trend in nitrogen tissue content of a wild *Gracilaria vermiculophylla* population. Mean ± S.E (n = 5).

content, NO_3^- uptake was diminished, with most of the N originating from NH_4^+. The same decrease in NO_3^- uptake rates with high N tissue content was observed for *U. rigida* (Naldi and Viaroli, 2002). On the other hand, the superior efficiency in NH_4^+ uptake was maintained during the continuous high levels of N enrichment. This was reported before for *G. tickvahiae* (Fujita, 1985). A possible explanation for this may be the previously referred energy savings when taking up ammonia. Moreover, when comparing the single N source addition treatments, the total N in *Gracilaria*'s tissue was significantly higher with NH_4^+ *vs* NO_3^- enrichment. The same was observed for *G. pacifica* (Naldi and Wheeler, 1999) and *G. tikvahiae* (Teichberg et al., 2008).

The low mean uptake performance across the 48 h period, when compared to the initial 4 h experiment, may be attributed to the inclusion of diel changes in the uptake processes. Uptake efficiencies were generally lower during dark periods, as expected, due to the lowest photosynthetic rates of seaweeds in absence of light (Lobban and Harrison, 1997). However, NH_4^+ dark uptake efficiencies were always above 61%, except for the treatment with the highest concentration. Nonetheless, even under that condition, NH_4^+ uptake was close to 30%. Effective NH_4^+ uptake during dark periods was also reported for *G. tikvahiae* (D'Elia and DeBoer, 1978) and *Porphyra dioica* (Pereira et al., 2008).

The 48 h period of this experiment was not enough to detect differences in the growth rates of *Gracilaria* under different treatments. However, in the comparison of treatments with only NH_4^+ or NO_3^- added, *G. vermiculophylla* seemed to have a tendency for higher growth with NH_4^+. *Gracilaria* species seem very versatile in their preferred N chemical form for growth. Some, like *G. tikvahiae* (DeBoer et al., 1978), have shown to grow better with NH_4^+ while others had no apparent preference for either NH_4^+ or NO_3^- (Lapointe and Ryther, 1978). For the determination of this relation, longer time course experiments are necessary.

4.1. Ecological relevance

High uptake rates during periods of high N availability give fast growing species, like *G. vermiculophylla*, an advantage over the slow growers. As a result, the populations of slow growing macrophytes, like seagrasses, can be negatively impacted by occasional blooms of those opportunistic species (Duarte, 1995; McGlathery et al., 2007; Naldi and Viaroli, 2002; Ralph et al., 2006). Several field studies with *G. vermiculophylla* found higher nitrogen tissue uptake and accumulation with higher availability of this nutrient in ambient seawater (Thomsen et al., 2006; Tyler and McGlathery, 2006). Although they did not include simultaneous water analysis, our observations also point that way. However, none of these field studies focused on this species ability to cope with different N sources. The latter may be an important assessment for understanding species abundance in estuarine areas, like Ria de Aveiro lagoon, that receives N inputs of multiple chemical species and from various sources. The ability of *G. vermiculophylla* to considerably take up NH_4^+ and NO_3^- under a wide range of concentrations, as observed in this study, may thus be one of the factors responsible for the observed dominance and fast proliferation of this non-indigenous algae species in estuarine environments (Abreu et al., submitted for publication; Thomsen et al., 2006; Thomsen et al., 2009). In order to understand the importance of nutrient dynamics in the apparent shift of dominance of *Z. noltii* to *G. vermiculophylla* in Ria de Aveiro lagoon (Silva et al., 2004), experimental studies directly comparing the eco-physiology responses of these two key estuarine organisms are necessary.

Another aspect that should be taken into account is the role of *G. vermiculophylla* as a natural biofilter of estuarine areas like Ria de Aveiro lagoon. In this study, there was clearly seasonal nitrogen accumulation in *G. vermiculophylla* tissue; this pattern is in agreement with the fluctuations of the main source of nitrogen (nitrate) in the lagoon (Lopes et al., 2007). If one considers the biomass of *G. vermiculophylla* thriving in those mudflats around 40 g^{-2} (Silva et al., 2004) and the

mean nitrogen tissue accumulation observed in this study (4.4% N, DW), it is possible to estimate a sink of nitrogen in the order of 1.76 g of nitrogen per square meter. Moreover, and considering the potential commercial value of *G. vermiculophylla* (Sousa et al., 2010), if this biomass was sustainably exploited, the removal of nitrogen from the lagoon would be really effective. So, the bio-extractive role of *G. vermiculophylla* on this ecosystem could be considered in the management of eutrophication as undertaken in other geographical areas (He et al., 2008; Jiang et al., 2010; Yang et al., 2006; Zertuche-González et al., 2009).

4.2. Potential for biofilter in IMTA systems

In this study we used a range of concentrations that might be expected in nutrient enriched aquaculture systems, including one with extreme values of nitrogen. *G. vermiculophylla* was able to cope (positive growth and N uptake performance) with all the ammonium and nitrate concentrations tested, independently of the addition of both or single N chemical species. However, the uptake performance of ammonium was superior to the one of nitrate; even at the highest N concentration tested *G. vermiculophylla* removed 63% of the ammonium input. In addition to the better uptake performance, it seemed that the nitrogen tissue accumulation would be enhanced in water enriched with ammonium. In addition, the ability of *G. vermiculophylla* to efficiently take up ammonium during dark periods, besides increasing the overall biofiltration process, might give a competitive advantage over epiphytes, as these may have limited dark N uptake (Hanisak, 1990). This high efficiency in ammonium uptake is advantageous for a seaweed biofilter since it can compete with the costly bacterial processes currently in use in intensive recirculation systems (Crab et al., 2007; Neori et al., 2004). A direct comparison between these two biofiltration processes has shown that a seaweed biofilter can outperform bacterial biofiltration efficiency (Cahill et al., 2010). Our study also showed that *G. vermiculophylla*, when supplied with nitrate alone, was also efficient (ca. 47%) in removing this nutrient. Therefore, its application as a biofilter in fish farms with a higher excretion of nitrate (semi-intensive systems), which normally present residual values of ammonium is also pertinent.

The experimental time scale of this study (4 h between media changes) is not sufficient to predict the effective success of a *G. vermiculophylla* biofilter system in all commercial fish farm operations. However, it may serve as an indicator to the manipulation of the residency time of the effluent in the seaweed biofilter system. Depending on the purpose of the company, whether being seaweed biomass production or nutrient uptake efficiency, other factors like the stocking density of the seaweed in the tanks will also be critical to the design of the biofiltration system (Abreu et al., 2011; Neori et al., 2004).

All of these features suggest that land-based aquacultures may mitigate their negative nutrient release to the environment by testing integrated systems based in the biofiltration capacity of *G. vermiculophylla*.

Acknowledgments

The first author acknowledges Y. Abdu, Dr. Jang Kim and Dr. Qiaohan Wang for the assistance at the Seaweed Marine Biotechnology Lab (University of Connecticut, Stamford Campus) and Dr. George Kraemer for the helpful suggestions throughout the experiments. The guidance of Mr. Dennis Arbige and Dr. Claudia Koerting during the water analysis process at the University of Connecticut (Avery Point Campus) is truly appreciated. The English review by Dr. Emma Seale is also acknowledged. The stay of Maria H. Abreu in USA was supported by the Portuguese Foundation for Science and Technology (FCT) with a PhD scholarship (ref: SFRH/BD/21363/2005) and the program POPH/FSE. R. Pereira was also supported by a fellowship from FCT through program POCI 2010, with the support of FEDER and FSE (SFRH/BPD/36451/2007). **[SS]**

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