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Article *in* European Journal of Phycology · February 2008

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Online Publication Date: 01 February 2008
To cite this Article: Pereira, Rui, Kraemer, George, Yarish, Charles and Sousa-Pinto, Isabel (2008) 'Nitrogen uptake by gametophytes of **Porphyra dioica** (Bangiales, Rhodophyta) under controlled-culture conditions', European Journal of Phycology, 43:1, 107 - 118
To link to this article: DOI: 10.1080/09670260701763393
URL: http://dx.doi.org/10.1080/09670260701763393

Eur. J. Phycol., (2008), **43(1)**: 107–118

Nitrogen uptake by gametophytes of *Porphyra dioica* (Bangiales, Rhodophyta) under controlled-culture conditions

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(Received 15 May 2007; accepted 23 October 2007)

Aspects of the nutrient-uptake physiology of *Porphyra dioica* (Brodie *et* Irvine) from Porto, Portugal were investigated under laboratory conditions. The capacity for uptake and accumulation of nitrogen (N) by *P. dioica* was determined for two different N sources, ammonium (NH_4^+) and nitrate (NO_3^-). The influence of the light–dark cycle and of the simultaneous presence of NH_4^+ and NO_3^-, as well as the effects of phosphorus (P) enrichment, on the growth, nutrient uptake, and accumulation were also evaluated. *Porphyra dioica* was able to take up, accumulate, and grow equally well using both sources of nitrogen when presented separately. The photosynthetic pigment levels increased significantly with the increase of the availability of N, for both sources. The chlorophyll *a* content was higher in thalli that used NO_3^- as source of N, while this difference was not seen for phycobiliprotein content. When both N sources were available ($NO_3 : NH_4 = 6 : 1$), *P. dioica* preferentially removed NH_4^+, with a clear diurnal difference. During the light period, the algae removed 70% of the NH_4^+ available, while only 35% was removed during the dark period. Phosphorus enrichment did not influence the growth rate or the amount of P removed from the medium, suggesting a limited capacity to store P. These results indicate that *P. dioica* is a good candidate for application in an integrated multi-trophic aquaculture (IMTA) system.

Key words: cultivation, IMTA, nitrogen, nitrogen uptake, phosphorus, *Porphyra dioica*, Rhodophyta

Introduction

Porphyra is one of the most important cultured seaweeds in the world, with 1.4 million metric tons of *Porphyra* (nori) produced in 2004, valued at 1.34 billion USD (FAO, 2006). This is surpassed only by the production of the kelp, *Laminaria japonica* (4.5 million metric tons, valued at 2.75 billion USD) and *Undaria pinnatifida* (2.5 million metric tons, valued at 1.02 billion USD; FAO, 2006). Data from FAO (2006) show that production of *Porphyra*, by weight, represents 12.5% of the world's seaweed mariculture, which in turn, represents 23% of the world's total production of marine organisms. *Porphyra* is used primarily for food, but also as a source of the red pigment *r*-phycoerythrin (Mumford & Miura, 1988). However, the genus has much more potential and can be used as an experimental system for applied and basic research (Sahoo *et al*., 2002).

Due to its high surface/volume ratio, *Porphyra* spp. are fast growing, as well as being capable of rapid assimilation of N and P. This fact, together with its economic value, makes the genus one of the most promising for bioremediation and Integrated Multi-Trophic Aquaculture applications (IMTA, *sensu* Chopin, 2006; Kraemer & Yarish, 1999; Neori *et al*., 2004). Preliminary studies by Chopin *et al*. (1999) demonstrated that *P. yezoensis* and *P. purpurea* respond to high nutrient levels in areas of salmon aquaculture and intense scallop dragging by incorporating additional N into tissues.

Successful agriculture depends on an understanding of the ecology and physiology of vascular plants. In the same way, successful mariculture depends on having extensive knowledge of the biology and physiology of the marine algae and how factors important to their growth can be manipulated to improve yields (Lobban & Harrison, 1994; Craigie & Shacklock, 1995). As Chopin *et al*. (1999) pointed out, the efficacy of different species of *Porphyra* as nutrient scrubbers must be compared to select the species or cultivar best suited for bioremediation.

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ISSN 0967-0262 print/ISSN 1469-4433 online/08/010107–118 © 2008 British Phycological Society
DOI: 10.1080/09670260701763393

Recently, Kraemer *et al.* (2004) and Carmona *et al.* (2006) compared nutrient uptake by native Northeast US species with Asian species in short- and long-term experiments, respectively. Essentially, those studies showed that several north-eastern American species of *Porphyra* can perform as well as or better than the Asian species presently used in aquaculture. An interesting complement to this is the work of Blouin *et al.* (2006), who compared the taste and textural qualities of *P. amplissima* and *P. umbilicalis* (two native Atlantic species of *Porphyra*) with those of *P. yezoensis* (Asian species). They reported no significant differences between the species in their taste acceptability for human consumption.

Porphyra dioica (Brodie *et* Irvine), a species native to the eastern North Atlantic, also appears to be a good candidate for aquaculture applications (Pereira *et al.*, 2006). This species grows rapidly under a wide range of temperatures and photoperiods. Young blades are vegetatively formed in the basal portions of adult blades (Pereira *et al.*, 2006), a form of vegetative propagation/perennation never described for this genus, but ideal for a periodically harvested crop.

In IMTA conditions, N will occur as ammonium (NH_4^+) and nitrate (NO_3^-). Nitrate results from bacterial nitrification, usually by a bacterial biofilter, while NH_4^+ will be derived from excretion by the animal component of the IMTA system. Seaweed species may differ in their relative preference of these two sources of N (D'Elia & DeBoer, 1978; Hanisak, 1983). A species capable of growing equally well with those two N sources will have advantages in IMTA systems.

Another important aspect of IMTA algal applications is the daily cycle of nutrient uptake. Seaweeds, as photosynthetic organisms, are likely perform differently under light or dark periods. To better estimate the overall efficiency as nutrients removers, knowledge of N uptake during the dark periods is required. Differences in NH_4^+ and NO_3^- uptake during a diurnal cycle were shown for phytoplankton (Glibert & Garside, 1992). Cohen & Neori (1991) also showed that ammonia uptake rate of *Ulva lactuca* decreased during the night. A diel rhythm of phosphate uptake for three species of microalgae, in P-limited conditions, was also reported by Ahn *et al.* (2002).

Nitrogen is the primary 'limiter' of algal growth in temperate marine regions, but at times phosphorus may also be limiting (Howarth, 1988; Krom *et al.*, 1991). These are also the main nutrients released by fish aquaculture. N and P in mariculture effluents occur at a ratio of ca. 7 : 1 (Chopin *et al.*, 1999), although this may vary according to fish species and diet composition. Considering that N will be available far in excess in fish-aquaculture effluents, it is necessary to verify whether any benefits would result from P enrichment.

This work provides information regarding the nutrient-uptake efficiency of *P. dioica*. The main objective was to determine how well this species can cope with the levels of N found in aquaculture effluents. Other objectives were to determine how the diurnal cycle and the simultaneous presence of NH_4^+ and NO_3^- in the medium, influences the nutrient uptake. A further objective was to determine the effects of P enrichment on the growth of the gametophytes of this species.

Materials and methods

Young gametophytes of *P. dioica* 1–3 cm long and approximately 4 weeks old were used for these experiments, except when stated otherwise. Isolates were obtained from a conchocelis culture derived from tissue collected in Porto, Portugal (41°19′37″N; 8°45′40″W) in September 2000. This culture, strain PD2-1, was established and maintained in culture in the Marine Biotechnology Laboratory, University of Connecticut, Stamford, CT, USA. Induction of conchosporangia and new gametophytes has been described elsewhere (Pereira *et al.*, 2004).

Conditions common to all treatments were temperature (15°C), photon flux density (PFD, 150 μmol photons $m^{-2} s^{-1}$), and photoperiod (12 : 12-h, light–dark cycle), with light provided by cool-white fluorescent tubes. Gametophytes of *P. dioica* were grown in 1-l flasks with 1-l of von Stosch's enriched seawater (Ott, 1965) modified to provide the desired NO_3^-, NH_4^+ and phosphate concentrations. The algae were always acclimated for 1 week to the experimental temperature, PFD and photoperiod conditions.

Ammonium vs nitrate uptake

In this experiment, the algae had either NO_3^- or NH_4^+ as source of N. Dissolved inorganic nitrogen (DIN) was added as $NaNO_3$ or NH_4Cl to obtain concentrations of 25, 75, 150 and 300 μM of N. For the NO_3^- enrichment, there was also an additional 500 μM treatment. Phosphate (PO_4^{3-}) was added as $Na_2HPO_4 \cdot 12H_2O$. The final P concentration in the medium was 3.1, 7.3, 12.3, 27.3 and 30.6 μM for each N-enrichment treatment, respectively, giving a constant average molar N : P ratio of 10 : 1. Three replicates per N source and concentration were used. The stocking density of the cultures was 0.3 ± 0.05 g fresh weight (fw) l^{-1}, and the medium was replaced twice each week. Growth was measured by the change in fw biomass in each flask and at each time point the stocking density was reduced to the initial value. Tissue samples were collected for pigments and carbon, hydrogen and nitrogen analysis (CHN). Each time the medium was changed, 100 ml of the medium was filtered and frozen (−20°C) for water analysis. The experiment lasted 3 weeks, and all tissues were acclimated one week in von Stosch's enriched

seawater (with NO_3^- as N source and 10 : 1 N : P ratio) under the common experimental temperature, PFD and photoperiod regimes. Flasks (1-l volume) with 1-l of modified von Stosch's enriched seaweed water (Ott, 1965) were used for this experiment.

Diel nitrogen uptake studies

For this experiment, algae were grown in 1-l flasks with 0.8 l of modified von Stosch's enriched seaweed water (Ott, 1965). The NO_3^- and NH_4^+ were present simultaneously in the medium, although in different concentrations. Dissolved inorganic nitrogen was added as $NaNO_3$ and NH_4Cl to obtain concentrations of 300 µM NO_3^- and 50 µM NH_4^+, respectively. Phosphate $\left(PO_4^{3-}\right)$ was added as $Na_2HPO_4 \cdot 12H_2O$ to obtain a concentration of 5 µM $\left(PO_4^{3-}\right)$. The nutrient concentrations were chosen in accordance to the typical concentration in the effluent of Great Bay Aquaculture, LLC (Portsmouth, NH, USA, C. Yarish, unpublished data).

Three replicates per condition were used. The stocking density of the cultures was 0.8 ± 0.05 g fw l^{-1} and the medium was renewed every 3 hours. Growth was measured by the change in biomass (fw) in each flask every 3 hours, when the medium was renewed. Medium changes and fw measurements were performed within the walk-in environmental chamber. During the dark period, medium changes were performed under 5–7 µmol photons $m^{-2}\,s^{-1}$ of red light. At the end of each growing period, i.e. every 3 hours, water samples were collected, filtered, and frozen (−20°C) for analysis.

Phosphorus enrichment studies

Young gametophytes of *P. dioica* used for these experiments were 2–4 cm long and 5 weeks old. The stocking density used in this experiment was 0.4 ± 0.05 g fw l^{-1}. Nitrate, as the sole source of N, was provided at 171 (±5.0) µM. Phosphate concentration varied to obtain N : P ratios of 3 : 1, 2 : 1, 1 : 1 and 1 : 2. The P concentrations were 60 (±4.9), 84 (±8.5), 140 (±5.0) and 400 (±39.2) µM of $\left(PO_4^{3-}\right)$, for each N : P ratio, respectively. Growth was measured by the change in biomass (fw) in each of the three replicate flasks – 1-l flasks with 1-l of modified von Stosch's enriched seaweed water (Ott, 1965). The medium was replaced twice per week. Each time the medium was changed, 100 ml was filtered and frozen (−20°C) for analysis. The experiment ran for 4 weeks.

Water analysis

Samples of 'spent' medium were analysed for inorganic N and P at the Center for Environmental Science & Engineering (CESE), University of Connecticut, Storrs, CT, using a Four Channel Auto Analyzer equipped with High-Sensitivity Seawater Cartridges (Lachat-QuikChem AE Ion Analyzer, Hach Company, Loveland, Colorado). The CESE is an US Environmental Protection Agency certified laboratory. For NO_3^- analysis, nitrate is reduced to nitrite at pH 7.5 in a copperized cadmium column. The nitrate reduced to nitrite, plus any free nitrite present, reacts under acidic conditions with sulfanilamide to form a diazo compound that couples with N-1-naphthylethylenediamine dihydrochloride to form a reddish-purple azo dye that is measured spectrophotometrically at 520 nm. For NH_4^+, the method uses the Berthelot reaction, in which a blue-green coloured complex is formed and measured at 660 nm. Phosphate was quantified following the method of Murphy & Riley (1962) for the determination of ortho-phosphate. The reduced blue phospho-molybdenum complex is assayed at 880 nm.

CHN analysis

The material collected during the experiments was dried in an oven at 50°C and later ground using an automatic grinder (Model MM200, Retsch & Haan, Germany). The percentages of carbon, hydrogen and nitrogen in the tissue were determined on a dry weight (dw) basis, using a CHN analyzer (Series II, CHNS/O 2400 Analyzer, Perkin Elmer Analytical Division of E.G. & G, Wellesley, MA, USA).

Pigment analysis

The algal tissue for pigment analysis was frozen immediately after collection and stored at −20°C. The analysis of phycobiliprotein (PBP) concentration was performed on aqueous crude extracts, following the method described by Beer & Eshel (1985) with some modifications. Samples of 0.03–0.1 g fw of tissue were ground, using mortar and pestle, with 5 ml of 0.1 M phosphate buffer (pH 6.8) and sand. The extraction was done at cold temperature, in an ice tray, in dim light, and the extracts held in the dark at 4°C overnight. They were then centrifuged at 10,000 *g* for 20 min and the supernatants were used for PBP determinations. Light absorption was measured using a Perkin Elmer, spectrophotometer (UV/VIS spectrophotometer Lambda 20, Perkin Elmer Analytical Division of EG & G, Wellesley, MA, USA).

For chlorophyll *a* (Chl *a*) analysis, we followed a procedure adapted from Chapman (1988). Briefly, the pellet from the phycoerythrin extraction was collected, 5 drops of a 1% $MgCO_3$ solution in distilled water were added, and the material ground in 4 ml, 90% acetone. After centrifuging for 20 min at 10,000 *g*, the supernatant was decanted and the extraction procedure repeated on the pellet. The supernatants from the two extractions were combined and the absorbance at 665 nm measured using a Perkin Elmer spectrophotometer (UV/VIS spectrophotometer Lambda 20, Perkin Elmer Analytical Division of EG & G, Wellesley, MA, USA).

Statistical analysis

Means and standard deviations were calculated for all treatments based on three replicates. Differences among treatments were tested for significance using two-way ANOVA. Multiple post-hoc comparisons among means were tested using the Student Newman Keuls (SNK)

test. In all cases, the null hypothesis was rejected at the 5% significance level, according to Sokal & Rohlf (1995).

Results

Ammonium vs nitrate uptake studies

The specific growth rate of the gametophytes of *P. dioica* was significantly influenced ($p < 0.01$) by the nitrogen concentration in the medium for both N sources. The growth rate reached a maximum of 25% fw day^{-1} in the presence of 300 µM N (Fig. 1) and did not increase significantly ($p > 0.05$) from 300 to 500 µM of NO_3^-. The nitrogen source (ammonium vs nitrate) did not affect growth rate ($p > 0.05$) and in both cases followed the Michaelis–Menten model.

The N content of the plants increased significantly ($p < 0.05$) and linearly with the increase in the N concentration (Fig. 2). However, the form of N did not affect tissue N content ($p > 0.05$). The highest N content was 4.9% dry weight (dw), recorded at the highest concentration tested, 500 µM of NO_3^-. If growth rate of the algae was plotted against their N content (Fig. 3), the data once again fitted the Michaelis–Menten model. Thalli with 4% tissue N reached a maximum growth rate of 25% fw day^{-1}. This was true for both N sources.

The carbon content of the tissue did not vary significantly ($p > 0.05$) with the different N concentrations or sources, with values between 37 and 39.5%. On the other hand, the C : N ratio decreased significantly ($p < 0.05$) with the increase in the N concentration in the medium. The

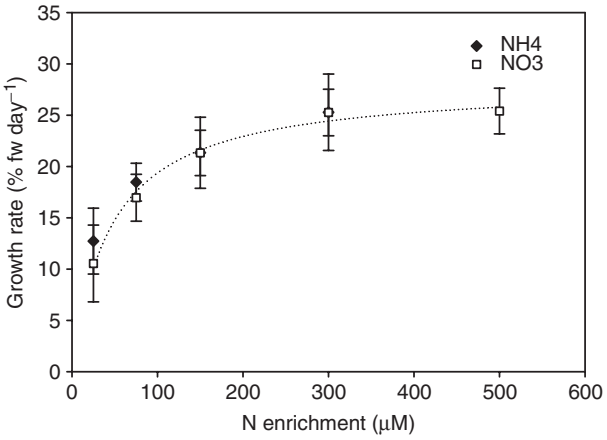

Fig. 1. Relationship between growth rate of *Porphyra dioica* and N enrichment with two N sources: NH_4^+ (◆) and NO_3^- (□). The dotted line (….) shows the Michaelis-Menten model fitted to the NO_3^- data. The r^2 is 0.990 for NO_3^- and 0.970 for NH_4^+. Other conditions: 15°C, 12 : 12-h, light–dark cycle and 150 µmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Symbols are the means of three replicates per treatment.

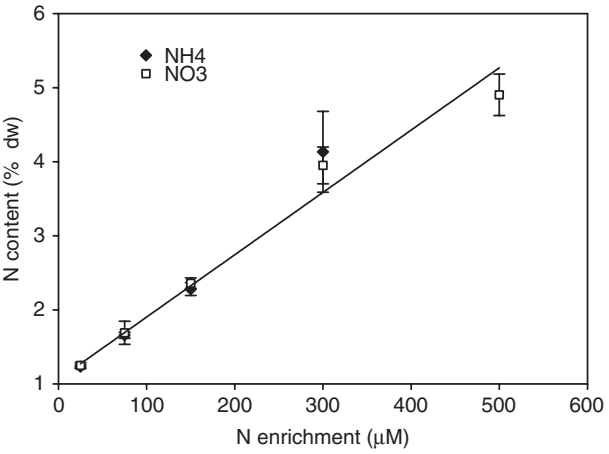

Fig. 2. Nitrogen content of gametophytes of *Porphyra dioica* under different N concentrations and two N sources: NH_4^+ (◆) and NO_3^- (□). The r^2 of a linear equation fitted to the lines is 0.976 for NO_3^- and 0.999 for the NH_4^+. Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle and 150 µmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Symbols are the means of three replicates per treatment.

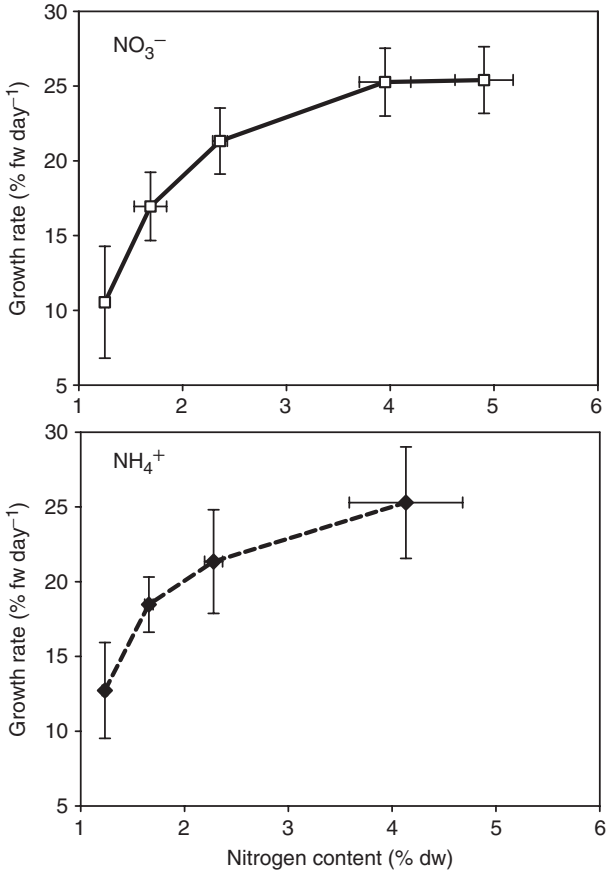

Fig. 3. Relationship between growth rate and N content of gametophytes of *Porphyra dioica*, under different N concentration and two N sources: NH_4^+ (◆) and NO_3^- (□). The r^2 is equal to 0.913 for NO_3^- and 0.929 for NH_4^+. Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle and 150 µmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} of stocking density. Symbols are the means of three replicates per treatment.

decrease in the tissue C : N ratio was similar for both N sources, from 35 to 11, at 25 μM and 300 μM, respectively. With 500 μM of NO_3^- the tissue C : N ratio was 8.8.

The amount of N removed from the medium increased linearly with the increase in the N concentration up to 300 μM, for both N sources (Fig. 4) and the ANOVA indicated no significant differences between the two N sources ($p > 0.05$). In the NO_3^--enrichment experiment, where we had an additional condition with 500 μM of N, the removal did not increase as much as, for instance, from 150 to 300 μM. With a 100% N increase from 150 to 300 μM, removal increased by 98%, while removal increased by only 24% with a 67% increase in N availability (300 to 500 μM). The increase in the N content of the thalli was also smaller from 300 to 500 μM of NO_3^- (Fig. 2).

At all concentrations, for algae cultured on either N source, the DIN in the medium appeared to have been totally removed by the seaweeds after 4 days (Fig. 4). However, the percentage of N incorporated into new tissue (Fig. 5) varied between different N concentrations of the medium for both N sources. In medium containing 25 μM N, all the N removed from the medium was incorporated into new tissue. At higher concentrations only a fraction of the N removed was incorporated into new tissue. *Porphyra* with 75 μM of N available were able to incorporate into new tissue approximately 75% of the N removed. Furthermore, *Porphyra* in the treatments with 150 and 300 μM incorporated 63 and 65%, respectively and thalli grown with 500 μM of NO_3^- incorporated 47% of this amount.

The uptake of (PO_4^{3-}) varied with the concentration available as the amount of (PO_4^{3-}) removed from the medium increased linearly with the increasing availability in the medium (Fig. 4). The percentage of available P removed from the medium decreased progressively from 88 to 70% when the NO_3^- concentration was reduced from 25 to 300 μM. When NH_4^+ was the only available N source, the (PO_4^{3-}) uptake was similar to that in the medium with NO_3^-, but did not present the same decreasing trend. In this case (PO_4^{3-}) uptake was 85, 95, 92 and 70% when NH_4^+ concentrations were 25, 75, 150 and 300 μM, respectively.

The levels of PBP and Chl *a* increased significantly ($p < 0.05$) with an increase in N availability in the medium. PBP concentration was not influenced by the N source available ($p > 0.05$; Table 1). The SNK test revealed a significant increase in the PBP content with the increase in N concentration in the medium. The only exception, for both N sources, was the increase from 25 to 75 μM of N, which resulted in no significant effect on the PBP content. The maximum PBP content (4.8 mg g^{-1} fw) was observed when the alga was grown in medium containing 500 μM NO_3^-. Phycoerythrin (PE) and phycocyanin (PC) levels followed the same pattern as the total PBP (Table 1). However, the Chl *a* content of the tissue was significantly different between the two N sources, being higher in the thalli grown in medium containing NH_4^+ (Fig. 6).

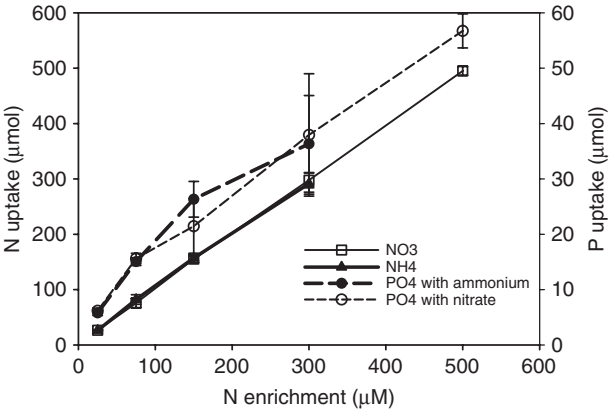

Fig. 4. Amount of N and P removed from the medium by gametophytes of *Porphyra dioica*, after 4 days under different N concentrations and two N sources. NH_4^+ uptake (◆),NO_3^- uptake (□), PO_4^{3-} uptake in medium with NH_4^+ (●) and PO_4^{3-} uptake in medium with NO_3^- (○). Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle and 15 μmol photons $m^{-2} s^{-1}$, 0.3 g fw l^{-1} stocking density. Symbols are the means of three replicates per treatment. P enrichment equal to 6, 15, 26, 51 and 60 μmol PO_4^{3-}, corresponding to the increase in N enrichment from 25 to 500 μmol.

Diel N-uptake studies

Only the uptake of NH_4^+ showed a significant ($p < 0.001$) diurnal influence (Fig. 7). During the

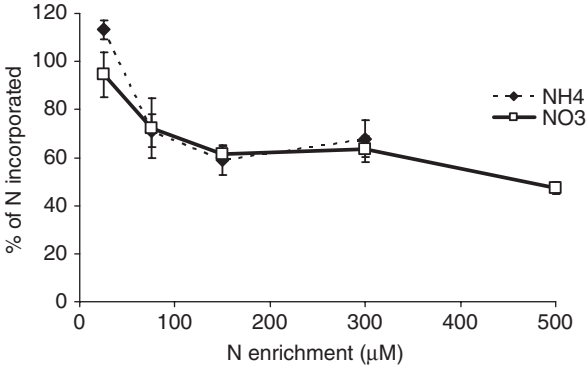

Fig. 5. Mean percentage of the N removed from the medium that was incorporated into new tissue by gametophytes of *Porphyra dioica* under different N concentrations and two N sources: NH_4^+ (◆) and NO_3^- (□). Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle and 15 μmol photons $m^{-2} s^{-1}$, 0.3 g fw l^{-1} stocking density. Symbols are the means of three replicates per treatment.

Table 1. Phycobiliprotein (PBP), Phycoerythrin (PE) and Phycocyanin (PC) content (mg g^{-1} fw) of the algae grown with NO_3^- or NH_4^+

		NO_3^- or NH_4^+ enrichment (μM)				
		25	75	150	300	500
NO_3^-	PE	0.20	0.31	0.78	1.99	2.84
	PC	0.07	0.14	0.45	1.17	1.93
	PBP	0.27	0.45	1.23	3.16	4.77
NH_4^+	PE	0.16	0.37	0.67	1.51	na
	PC	0.07	0.21	0.40	0.82	na
	PBP	0.23	0.58	1.07	2.33	na

na: the concentration of 500 μM NH_4^+ was not used, there are no PBP values for this treatment.

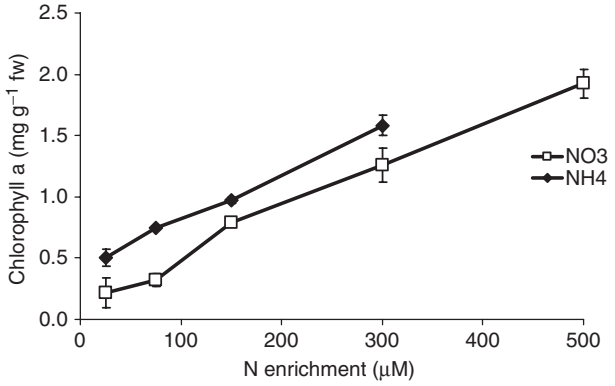

Fig. 6. Chlorophyll *a* content of thalli of *Porphyra dioica* under different N concentrations and two N sources: NH_4^+ (◆) and NO_3^- (□). Other environmental conditions: 15°C, 12 : 12-h, light–dark cycleand 150 μmol photons $m^{-2}s^{-1}$, 0.3 g fw l^{-1} stocking density. Symbols are the means of three replicates per treatment.

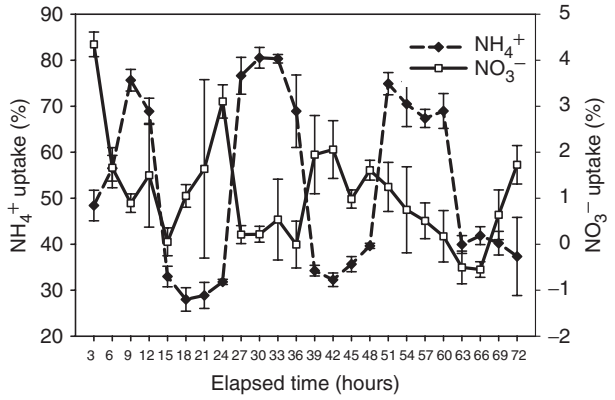

Fig. 7. Mean diel variations in uptake percentage of NH_4^+ (◆) and NO_3^- (□) by gametophytes of *Porphyra dioica*. Medium consisted of VSE with 50 μM NH_4^+, 300 μM NO_3^- and 35 μM of PO_4^{3-}. Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle and 150 μmol photons $m^{-2}s^{-1}$. Symbols are the means of three replicates per treatment. Shadowed areas in the x-axis represent the night periods.

light period, *P. dioica* removed 70% (± 9.6) of the available NH_4^+ from the medium, while removing only 35% (± 5.2) of the NH_4^+ during the dark period.

The percentage of NO_3^- removed from the medium was always much lower than the percentage of NH_4^+ and a maximum of 3% NO_3^- was removed at the end of the first night period (if we neglect the 4.3% in the first 3 hours of the experiment). Although Fig. 7 seems to reveal a tendency for higher NO_3^- uptake in the dark (average removal = 1.1%) than during the lighted period (average removal = 0.7%), the data are not statistically different ($p > 0.05$). Furthermore, the between-replicate variation in uptake was higher for NO_3^- than for NH_4^+. It was noted that the (PO_4^{3-}) uptake was not influenced by the day and night cycle remaining relatively stable throughout the experiment.

Phosphorus enrichment studies

During each culture period (3–4 days), *Porphyra dioica* appeared to remove almost 100% of the NO_3^- in the medium. The percentage of (PO_4^{3-}) removed decreased with increasing (PO_4^{3-}) concentration, corresponding with the decrease in the N : P ratio of the medium (Fig. 8). While the relative (% of available) (PO_4^{3-}) uptake varied, the absolute removal remained constant, from 47 to 51 μmol (PO_4^{3-}) per culture period.

The uptake of (PO_4^{3-}) fluctuated depending on the culture period, 3 or 4 days, before medium replenishment, although the average P-removal per day was similar (Fig. 9). More (PO_4^{3-}) was removed at the end of each 4-day period (61% on average) than at the end of each 3-day period (44% on average, for the 3 : 1 N : P ratio) (Fig. 9).

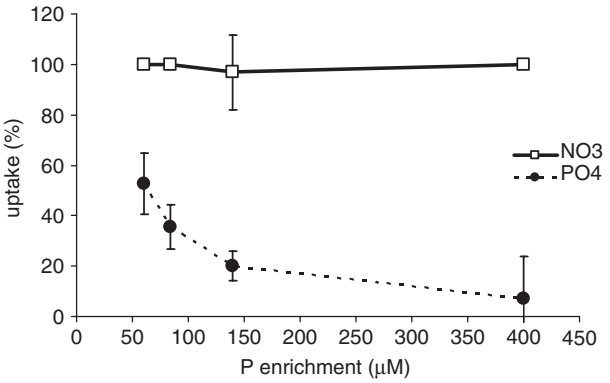

Fig. 8. Mean percentage uptake of NO_3^- (□) and PO_4^{3-} (●) by gametophytes of *Porphyra dioica* in different P concentrations. Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle, 150 μmol photons $m^{-2}s^{-1}$ and 171(± 5) μM NO_3^-. Symbols are the means of three replicates per treatment.

This was more evident at the highest N : P tested, but was true for all N : P ratios. Growth rates of *P. dioica* did not change significantly ($p > 0.05$) within the range of N : P ratios tested in this experiment, varying from 18.1 to 18.7% fw day^{-1}.

Discussion

Ammonium vs nitrate-uptake studies

Porphyra has the characteristics of an opportunistic species, with fast growth and the capacity to deal with high nutrient concentrations. The maximum growth rates of 25% recorded here are similar to those obtained for this species in previous experiments, whenever N was not limiting (Pereira *et al*., 2006). Carmona *et al*. (2006) recorded a maximum growth rate of 25% for *P. amplissima* and 18% for *P. yezoensis* and *P. umbilicalis* in conditions similar to the ones in our experiment. It is interesting to note that *P. dioica* is able to grow equally well using NO_3^- or NH_4^+ as the source of N when only one of these sources is available. Not all macroalgae can grow equally well on NO_3^- or NH_4^+ as studies on *P. yezoensis* (Wu *et al*., 1984; Amano & Noda, 1987) have demonstrated, that better growth and uptake rates were observed when NH_4^+ was the source of N, in comparison to NO_3^-. On the other hand, Hafting (1999) found that under a high PFD (160 µmol photons m^{-2} s^{-1}) *P. yezoensis* grew better when NO_3^- was the N source, whereas no differences were observed on growth under a low PFD (50 µmol photons m^{-2} s^{-1}).

The preference of one nitrogen form over another is dependent on the concentration used in the medium and on the environmental conditions. DeBoer *et al*. (1978) noted that *Gracilaria foliifera* grows better on NH_4^+ than on NO_3^- in laboratory conditions, whereas Lapointe & Ryther (1978) found that *Gracilaria tikvahiae* (when above a minimal daily nitrogen load) grows equally well on either N sources under high light in outdoor tanks. However it is possible, as suggested by Hanisak (1983), that some discrepancies in nutrient-utilization studies may be due to toxicity caused by high levels of the added nitrogen (NH_4^+ or organic N). For some seaweed, including: *Ulva lactuca* (Waite & Mitchell, 1972), *Hypnea musciformis* and *Macrocystis pyrifera* (Haines & Wheeler, 1978), concentrations of NH_4^+ over 30–50 µM can be toxic. Furthermore, Amano and Noda (1987) observed NH_4^+ toxicity effects on *P. yezoensis* only at concentrations above 30 ppm (ca 2.1mM), while Carmona *et al*. (2006) reported reduced growth of *P. umbilicalis* at 1.4 mM NH_4^+. However, in this study, that did not appear to be the case for *P. dioica*, at least at N concentrations up to 300 µM.

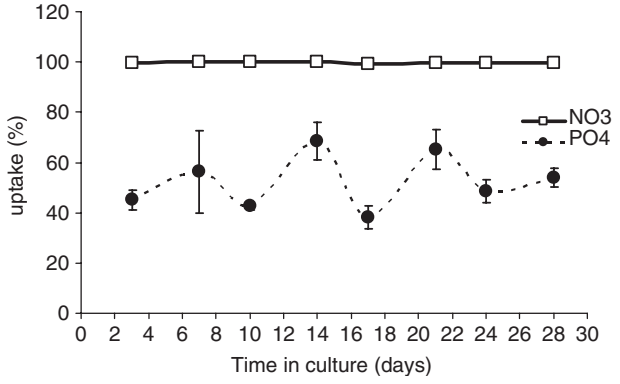

Fig. 9. Mean percentage uptake of NO_3^- (□) and PO_4^{3-} (●) by gametophytes of *Porphyra dioica* after each period of 3 or 4 days before medium replacement. Medium consisted of VSE with 178 µM NO_3^- and 58 µM of PO_4^{3-}, i.e., N : P = 3 for the data presented. Other environmental conditions: 15°C, 12 : 12-h, light–dark cycle and 150 µmol photons m^{-2} s^{-1}. Symbols are the means of three replicates per treatment.

The above data are relevant for the eventual application of these species in IMTA as NH_4^+ is the primary form of N resulting from fish metabolism. However, NH_4^+ concentrations must be controlled because they can be toxic for fish. Person-LeRuyet *et al*. (1995) reported that 40 mg l^{-1} of total ammonia-N ($NH_3 + NH_4^+$) was the LD_{50} for juvenile seabass, *Dicentrarchus labrax*. This is significant because NH_4^+ concentrations up of 207 µM (3 mg l^{-1}) have been recorded in fish effluents (Neori *et al*., 1996). Although most of the NH_4^+ will be transformed into NO_3^-, due to bacterial nitrification, seaweeds able to cope with high levels of NH_4^+ are advantageous for IMTA systems, reducing the amount of bacterial bioconversion necessary.

The N content of the thalli increased significantly with the increasing DIN availability in a way similar to that for growth rate. However, unlike growth rate, N content increased linearly and does not seem to reach an asymptote, at least up to 500 µM of NO_3^- or 300 µM of NH_4^+. In fact in this study, *P. dioica* was able to remove all N available in all concentrations and both N sources (Fig. 4).

The relationship between tissue N content and growth rate was non-linear. For both N sources, tissue N content greater than 4% dw did not correlate with higher growth rates (Fig. 3). This agrees with our prior experiments that employed similar N concentration and PFD (Pereira *et al*., 2006) and although the study was not designed for that purpose, our data suggest a critical N concentration (*sensu* Hanisak, 1983) for *P. dioica* of ca. 4% dw, as N contents above this value have no effect on the growth rate of

P. dioica. Other *Porphyra* species have been reported to have critical N levels similar to the one suggested by our results, for example, Wu *et al.* (1984) achieved maximal growth rate of *P. yezoensis* (11.6% day^{-1}) with a N content of 4.7% dw. Hafting (1999) also determined for *P. yezoensis* a critical N content of 0.4% fw (4.0% dw considering a fresh to dry ratio of 10 : 1), regardless of the N source (NH_4^+ or NO_3^-). However it should be noted that, Fujita *et al.* (1989) recorded critical N levels of $\leq 2.4\%$ dw and 3.0% dw depending on the N source, (NO_3^- and NH_4^+ respectively) for *Ulva rigida*.

The levels of the photosynthetic pigments Chl *a* and PBP increased in response to increases in nitrogen removal. The importance of PBP as N storage compounds in red algae will be discussed later. Other important pools of N in the tissue are the free amino acids (FAA) and mycosporine like amino-acids (MAA). MAAs constitute a small pool when compared with FAA, but its content can be 1.5-times greater than PBP content (Peinado, 2003).

Free amino acid composition is influenced by nutrient source and concentration (Bird *et al.*, 1982; Horrocks *et al.*, 1995). Nisizawa & Oofusa (1990) noted that the total pigment content is positively correlated with the organoleptic value of the tissue. Simultaneously, the important taste characteristics of *Porphyra* are correlated with its alanine, glutamic and aspartic acid contents (Nisizawa & Oofusa, 1990) and also inosinic and guanylic acids (Noda, 1975). Therefore, an increase in FAA content can reasonably be expected to accompany the observed increase in the tissue N and pigments content of *P. dioica* under high N concentrations. This expected difference in FAA content of the plants grown in higher N concentration can explain the lower percentage of N incorporated, if we consider the possibility of release of organic molecules.

Tyler *et al.* (1994) reported the release of dissolved organic nitrogen (DON) by *U. lactuca* during active growth. Furthermore, DON release has also been reported from phytoplankton (Collos *et al.*, 1992; Bronk & Ward, 1999). However, Naldi & Wheeler (2002) observed very little release of DON by *U. fenestrata* and *G. pacifica* using ^{15}N isotopes. These authors also reported that the concentration of FAA in the medium increased significantly only on the first day and only when NH_4^+ was the source of N. They suggested that the release of DON was probably due to the degradation of detritus rather than from live algal tissue. However, in our study no detritus was apparent in the culture flasks.

Another possible explanation for the difference between N removal and N incorporation is the volatilization of ammonia. The equilibrium between gaseous un-ionized ammonia (NH_3) and aqueous, ionized ammonium (NH_4^+) is strongly influenced by pH and it has been approximated that at pH 8.3 about 10% of the ammonia is NH_3, a value that increases to 50% at a pH 9.3 (Hargreaves, 1998). We know from previous studies (Pereira *et al.*, 2006) that *P. dioica* can deplete dissolved inorganic C and elevate the pH to 8.8. The higher growth rates at higher N concentrations may have caused increases in pH, with consequent volatilization of some of the ammonia, which was lost (and appeared to have been removed). This mechanism does not explain the same discrepancy for the NO_3^--enriched medium. Although our culture system was not axenic, all materials used were sterilized and denitrification by bacteria is unlikely to be significant. Further studies are needed to determine the fate of the missing nitrogen.

C : N ratios are generally higher when plants are grown under N limitation because of a decrease in proteins and an increase in carbohydrates, the so-called 'Neish effect' (1977). An inverse relationship between carbohydrates and N availability was reported by Gerard (1982) for *Macrocystis pyrifera* and by Rosenberg & Ramus (1982) in *G. foliifera* and *Ulva* sp. In the case of *P. dioica* the observed decrease in the C : N ratio was solely due to the increase in the nitrogen content of the seaweeds, since there was no significant difference in the carbon content of the thalli.

The PE content increased, on average, nearly 10-fold in thalli grown with 300 μM NO_3^- compared with those with 25 μM NO_3^-, furthermore, N source had no obvious influence on PE content. The maximum values of PE obtained in the study (2.85 ± 0.13 mg PE g^{-1} fw) are comparable to other *Porphyra* species, including: *P. linearis* (1.80 to 3.67 mg g^{-1} dw) (Conitz *et al.*, 2001), *P. amplissima* (0.67 ± 0.10 mg PE g^{-1} fw), *P. purpurea* (3.25 ± 0.35), *P. haitanensis* (0.73 ± 0.52 mg PE g^{-1} fw) for and *P. katadai* (0.70 ± 0.08 mg PE g^{-1} fw) (Carmona *et al.*, 2006). The increase in PC with the increasing DIN concentration was greater when thalli were supplied with NO_3^- than with NH_4^+. From 25 to 300 μM, the PC content of the thalli grown with NO_3^- increased 15-fold, while that of the thalli with NH_4^+ increased 10-fold. This observation is contrary to the findings by Amano & Noda (1987).

Changes in the PBP content were expected, since these pigments constitute important N-storage compounds in red algae (Bird *et al.*, 1982). Phycoerythrin comprises 20% of total N of *G. tikvahiae* (Lapointe & Duke, 1984). In *G. pacifica* (Naldi & Wheeler, 1999),

PE represented 5–6% of total N. The importance of different N compounds to the global storage pool varies with species. In the case of *P. dioica*, the PE content is very low in comparison to those of *Gracilaria* species and based on a nitrogen-to-protein conversion factor of 4.92 (Lourenço *et al*., 2002), the highest N content recorded for *P. dioica* in our work, 4.9% dw, corresponds to a protein content of 241 mg g^{-1} dw. Considering a PE content of 2.85 mg g^{-1} fw and a dry-to-wet weight ratio of 0.22, the PE content corresponds to only 0.26% of the total N. Applying the same calculation to data from Carmona *et al*. (2006) for *P. purpurea* (N content 6% dw, PE content 3.44 mg g^{-1} fw and assuming the same dry-to-wt weight ratio) PE corresponds to 0.26% of the total N. Clearly for these two species of *Porphyra* PE may not be such an important N-storage compound.

Diel N-uptake studies

The results of the diel uptake experiment indicated that *P. dioica* prefers NH_4^+ to NO_3^- when both sources are present, at the concentrations used here. Although there was 6-fold more NO_3^- available, the relative amount of NH_4^+ removed was 30 to 100-fold higher.

Nitrate uptake by phytoplankton is inhibited by NH_4^+ concentrations as low as 1 μM (Conway, 1977), although some seaweeds (especially kelp) are able to take up NO_3^- and NH_4^+ simultaneously and at the same rate (Harrison *et al*., 1986). In contrast, other seaweeds take up NH_4^+ preferentially over NO_3^-, with NH_4^+ inhibiting the uptake of NO_3^- (Harrison & Hurd, 2001). Partial inhibition of NO_3^- uptake by NH_4^+ is common in seaweeds (D'Elia & DeBoer, 1978; Haines & Wheele, 1978; DeBoer, 1981). D'Elia & DeBoer (1978) reported that *Neoagardhiella baileyi* and *Gracilaria foliifera* (Rhodophyta) preferred NH_4^+ over NO_3^-, even when plants were preconditioned on NO_3^- as the sole N source, this was also the case in this study with *P. dioica*. D'Elia & DeBoer (1978) also reported that NO_3^- uptake was suppressed at 5 μM NH_4^+, but simultaneous uptake occurred at lower concentrations. In our study, the uptake of NO_3^- by *P. dioica* was strongly affected, but still occurred at ca. 50 μM NH_4^+. Thomas & Harrison (1985) demonstrated that NH_4^+ only inhibited NO_3^- uptake by *P. perforata* for the first 10 to 20 minutes, thereafter NO_3^- uptake rates were independent of NH_4^+ concentration. They also found that this temporary inhibition does not occur if the blades were N-starved for 8 days.

Phosphorus enrichment studies

Nitrate uptake results during the N : P ratio experiment suggest that the algae were N-limited. All NO_3^- was removed from the medium by the end of each 3–4-day culture period, and the thalli were growing 18% fw day^{-1}, which was below the maximum growth rate obtained with higher NO_3^- concentrations. Despite this, it is interesting to note that the N : P-uptake ratio was constant for all N : P ratios. The percentage of uptake of (PO_4^{3-}) decreased with the increasing concentration, but the total amount of PO_4^3- removed was the same, at a N : P ratio of 5.75 ± 0.05. Hafting (1999) stated that *P. yezoensis* cannot store excess P over the range of loads tested (0.26 to 16.29 μmol day^{-1}). In our study, the increase in the concentration of (PO_4^{3-}) in the medium (with constant NO_3^- concentrations) was not reflected in the (PO_4^{3-}) uptake, suggesting lack of ability to store P (see Table 2).

In the NH_4^+ vs NO_3^- uptake experiment, the N : P uptake ratios increased from 8.8 to 18.7, with the increasing NO_3^- concentration from 25 to 500 μM. Since P was always available in excess, it is reasonable to assume that it was taken up according to the immediate needs of algal thalli. Therefore, a N : P ratio of 15 : 19 seems to be enough for maximum growth of *Porphyra dioica* when N is not limiting (Table 2). This N : P ratio is close to the 13 : 15 ratio, suggested by Hafting (1999) as the optimum for *P. yezoensis*.

Application to IMTA systems

The application of these results to the data available from the aquaculture industry will give an idea of the potential of *P. dioica* for IMTA systems. The estimated amount of P and N released by tons of fish produced per year is 7.0 and 49.3 kg, respectively (Chopin *et al*., 1999; McVey *et al*., 2002). Based on that estimate, a ton of fish would release on a daily basis around 19.2 g of P and 135.1 g of N. Considering the best results obtained in this study (141.5 μmol N and 16.2 μmol P removed per day by a culture with 0.3 g fw l^{-1} stocking density), we estimate that 20.5 kg of *P. dioica* would remove 100% of the N and 86% of the P released daily by ton of fish.

Porphyra dioica has the characteristics of a good species for biofiltration, as defined by Neori *et al*. (2004). It is capable of rapid growth and assimilation of significant amounts of inorganic nutrients. The maximum growth rate obtained in this study is one of the highest reported for *Porphyra* species. In addition, *P. dioica* demonstrated an equal capacity for removing NO_3^- and NH_4^+ from the culture medium. This is an important

Table 2. Summary of N- and P-uptake performance

Experiment	Growth rate (% day^{-1})	Daily load (μmol)			Daily uptake (μmol)			Daily uptake (μmol)[a]		
		N	P	N:P	N	P	N:P	N	P	N:P
PO_4^{3-}enrichment	18.1	51	16.9	3.0	51	8.9	5.7	38	6.7	5.7
	18.2	48	24.0	2.0	48	8.5	5.7	36	6.4	5.7
	18.7	48	40.1	1.2	47	8.1	5.8	35	6.1	5.8
	18.6	48	113.3	0.4	44	8.2	5.8	36	6.2	5.8
NO_3^- enrichment	10.5	7	0.9	7.9	7	0.8	8.8	7	0.8	8.8
	17.0	21	2.1	10.2	21	1.8	11.7	21	1.8	11.7
	21.3	42	3.5	11.9	42	2.7	15.6	42	2.7	15.6
	25.3	84	7.8	10.8	84	5.4	15.6	84	5.4	15.6
	25.4	143	8.7	16.4	142	7.6	18.7	142	7.6	18.7
Diel-uptake	15.5	2912[b]	77.4	37.6	430[b]	28.0	15.4	161	10.5	15.4

[a]Data normalized to 0.3 g fw l^{-1} stocking density.
[b]In this experiment the N load is the sum of NH_4^+ and NO_3^- available in the medium.
Uptake values are also the sum of NH_4^+ and NO_3^- uptake.

feature because, generally, intensive land-based aquaculture systems have bacterial biofilters that transform NH_4^+ to NO_3^-. Unless the seaweeds receive water directly from the fish tanks, most of the N available in the system will be in the form of NO_3^-. Furthermore, the presence of NH_4^+ does not appear to inhibit completely the uptake of the NO_3^-, another important advantage.

In conclusion, *P. dioica* is an excellent candidate for IMTA systems. Studies at a pilot scale should be carried out to confirm if the findings of this study can be extrapolated from laboratory experiments to a full field trial. Further studies to investigate the potential of this species for direct human consumption, and other uses (for instance replacement of animal protein in feed) are also needed. We consider it crucial that marine macroalgae with economic value are used to assure the economic sustainability of the IMTA systems.

Acknowledgements

R. Pereira was sponsored by the Portuguese Foundation for Science and Technology (FCT) through a postdoc grant (BPD 14840/2003). Support for this project was also provided by funding by Critical Technology Program of the State of Connecticut, the State Sea Grant College Programs of Connecticut and New York, and NOAA's National Marine Aquaculture Initiative. Equipment and technical support were generously provided by Perkin-Elmer Analytical Division of EG&G, Wellesley, MA. Many thanks to Dr D. Snellgrove, S. Kwitny and A. Semke who assisted in the University of Connecticut Marine Biotechnology Laboratory.

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