Faculdade de Ciências da Universidade do Porto

Departamento de Botânica

STUDIES ON THE ECOPHYSIOLOGY AND BIOCHEMISTRY OF *PORPHYRA DIOICA* BRODIE *ET* IRVINE IN CULTURE

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“The true art of discovery consists not in finding new lands
but in seeing it with new eyes”

Marcel Proust

Aos meus Pais
À Lígia

DECLARAÇÃO

Na elaboração desta dissertação, e nos termos do nº2 do artigo 8º do Decreto de Lei nº 388/70, os resultados de trabalhos já publicados foram totalmente aproveitados e fazem parte integrante de alguns capítulos desta dissertação.

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RESUMO

O género *Porphyra* é um dos géneros mais importantes de macroalgas marinhas utilizadas em aquacultura, tanto em valor monetário como em biomassa produzida. Segundo dados da FAO, organização das Nações Unidas, em 2001 foram produzidas 1132 milhões de toneladas de *Porphyra*, avaliadas em 1,2 biliões de dólares. Este valor de produção representa 16,3% do total de aquacultura de macroalgas, que por sua vez corresponde a 28,4% da produção total mundial de aquacultura (incluindo peixes, moluscos e crustáceos). Actualmente, as algas do género *Porphyra* são essencialmente usadas na alimentação humana e como fonte do pigmento ficoeritrina-r.

Este estudo foi pensado de modo a aumentar o conhecimento sobre a biologia de *Porphyra dioica*, uma das espécies de *Porphyra* mais comuns na costa Norte de Portugal. O primeiro objectivo era caracterizar o ciclo de vida desta espécie na natureza e no laboratório. A monitorização da percentagem de cobertura das populações naturais mostrou que *P. dioica* está presente durante todo o ano nesta zona. Os mesmos estudos revelaram uma maior percentagem de cobertura (23 e 66%) entre Fevereiro e Maio. De modo a caracterizar o ciclo de vida em laboratório, foram testados os efeitos da temperatura, intensidade luminosa e fotoperíodo, na germinação dos zigotosporos e no crescimento e reprodução da fase conchocelis. Os zigotosporos germinaram mais rapidamente a 15ºC e 25 µmol fotões m^{-2} s^{-1}. A taxa de crescimento dos conchocelis foi mais influenciada pela temperatura do que pelo fotoperíodo e intensidade luminosa. Nos três fotoperíodos testados a taxa de crescimento foi sempre mais elevada a 15ºC e com 25 a 75 µmol fotões m^{-2} s^{-1}. A frequência de formação de conchosporângios foi mais elevada a 15ºC em dia-curto, $8{:}\overline{16}$ h L:$\overline{D}$ e 25 a 75 µmol fotões m^{-2} s^{-1}, sendo praticamente inexistente em culturas de conchocelis em suspensão. As condições óptimas para formação de conchosporângios também se revelaram as melhores para libertação dos conchosporos após cerca de 18 semanas. O arejamento das culturas é condição essencial para a germinação dos esporos e obtenção de gametófitos. Não se observou formação de arqueosporos ou de qualquer outro tipo de esporos assexuais.

O segundo objectivo principal era determinar as condições óptimas para o crescimento dos gametófitos e aferir do seu potencial para uso em aquacultura integrada, como removedores de nutrientes. Nesse sentido, foi testada a influência da densidade de cultura (pf – peso fresco) e da intensidade luminosa na taxa de crescimento, produtividade e remoção de nutrientes. A taxa de crescimento máxima

(33% dia^{-1}) foi registada em culturas com 0,1 g pf l^{-1} e 150 a 250 µmol fotões m^{-2} s^{-1}. A taxa de crescimento diminuiu significativamente com o aumento da densidade da cultura, enquanto a produtividade mostrou uma tendência inversa. A 150 µmol fotões m^{-2} s^{-1} e com 1,5 g pf l^{-1}, foram produzidas 1,4 g pf l^{-1} $semana^{-1}$. A esta intensidade luminosa não se registou diferença significativa na produtividade a densidades entre 0.6 e 1,5 g pf l^{-1}. O conteúdo de azoto (N) das algas diminuiu com o aumento da densidade de cultura e da intensidade luminosa. Maior remoção foi registada nas culturas com 1,5 g pf l^{-1} e recebendo 150 µmol fotões m^{-2} s^{-1} (1,67 mg N dia^{-1}). Contudo, o N removido pelos talos a 50 µmol fotões m^{-2} s^{-1} foi estatisticamente igual ao removido pelas culturas a 150 e 250 µmol fotões m^{-2} s^{-1} à densidade de 1,0 g pf l^{-1}. A influência da temperatura e do fotoperíodo no crescimento e reprodução dos gametófitos foi também testada. As taxas de crescimento de *Porphyra dioica* foram significativamente afectadas por ambos os factores. A taxa de crescimento mais elevada (27,5% dia^{-1}) foi observada a 15ºC e 16:$\overline{8}$ h, L:$\overline{D}$. A formação de plântulas na parte basal de talos femininos não fecundados foi observada após 4 semanas em cultura. Após 7 semanas a biomassa produzida era resultante do crescimento destas novas lâminas, com taxas de crescimento entre 22,4 e 26,1% dia^{-1}. A capacidade de crescer e assimilar N em grandes quantidades foi testada para concentrações crescentes de N sob duas formas, nitrato (NO_3^-) e amónio (NH_4^+). A taxa de crescimento de *Porphyra dioica* aumentou linearmente com o aumento da concentração de N no meio, pelo menos até 500 µM NO_3^-. Os talos de *P. dioica* cresceram igualmente bem utilizando NO_3^- ou NH_4^+ como fonte de N, não se observando efeitos tóxicos do NH_4^+ nas concentrações testadas. Quanto aos ciclos diários de remoção de nutrientes, o NH_4^+ removido durante o período de luz foi o dobro do removido durante a noite. A remoção de NO_3^- aumentou ligeiramente durante a noite e a remoção de fosfato permaneceu constante. Nesta experiência constatou-se ainda que, na presença das duas fontes de N, *P. dioica* utiliza preferencialmente o NH_4^+.

Os resultados obtidos nestes trabalhos mostram que *Porphyra dioica* é uma boa candidata para aplicação em aquacultura integrada. Evidências para este facto são: 1) as suas altas taxas de crescimento, obtidas numa considerável amplitude de temperaturas e fotoperíodos; 2) capacidade de remover e acumular grandes quantidade de N, sob elevadas concentrações e em pelo menos 2 formas (NO_3^- e NH_4^+); 3) possibilidade de propagação vegetativa dos talos de gametófito. A aplicação desta espécie vai depender de futuros trabalhos em sistemas em maior escala e da aferição da qualidade e valores específicos da sua biomassa.

ABSTRACT

The genus *Porphyra* is one of the most important seaweeds in aquaculture, both in value and biomass. According with the Food and Agriculture Organization of the United Nations, 1.132 million metric tons of *Porphyra* were produced in 2001, valued at 1.2 billion USD. Production of *Porphyra* represents 16.3% of the world's seaweed mariculture, which in turn corresponds to 28.4% of the world's total mariculture production including fish, aquatic plants, molluscs and crustaceans. *Porphyra* is mainly used for human consumption and as source of the red pigment r-phycoerythrin.

This study was thought to increase the knowledge of the biology of *Porphyra dioica*, one of the most common *Porphyra* species in the North of Portugal. The first objective was to characterize the life cycle of the species, both in the field and in the laboratory. The monitorization of the percentage cover of the population in the field showed that *P. dioica* can be found throughout the year. Field studies showed higher percent cover, from 23 to 66%, during February through May. In order to follow the life cycle in the laboratory, the effects of temperature, photon flux density (PFD) and photoperiod on growth and reproduction were tested. The zygotospores germinated faster at 15ºC, and at 25 µmol photons m^{-2} s^{-1}. Growth rate of the conchocelis was affected by temperature and not by photoperiod. In the three photoperiods tested, growth rate was always higher at 15ºC, under 25 to 75 µmol photons m^{-2} s^{-1}. Conchosporangia formation was higher in 15ºC, short-day, 8:$\overline{16}$h, L:$\overline{D}$ cycle and 25 to 75 µmol photons m^{-2} s^{-1} and was almost non-existent in free floating conditions. Optimal conditions for conchosporangia maturation also promoted spore release after 18 weeks. Aeration is crucial for normal blade development. No archeospores or any other kind of asexual spores were observed.

The second main objective was to determine the optimal conditions for the growth of the gametophytes and to assess its potential for use in integrated aquaculture, as nutrient removers. The influence of stocking density and PFD on the growth, production and nutrient removal was tested. Maximum growth rates, up to 33% per day, were recorded with 0.1 g fw l^{-1} at 150 and 250 µmol photons m^{-2} s^{-1}. Growth rate decreased significantly with increasing stocking density, while productivity had an inverse trend. At 150 µmol photons m^{-2} s^{-1} and with 1.5 g fw l^{-1}, 1.4 g fw l^{-1} $week^{-1}$ were produced. At this PFD, there was no significant difference in production between 0.6 to 1.5 g fw l^{-1}. Nitrogen (N) content of the seaweeds decreased with increasing stocking

densities and PFDs. The maximum N removal was recorded at 150µmol photons m^{-2} s^{-1}, with 1.5 g fw l^{-1} stocking density (1.67 mg N day^{-1}). However, the N removed by thalli at 50 µmol photons m^{-2} s^{-1} was statistically equal to that at 150 and 250 µmol photons m^{-2} s^{-1}, at a stocking density of 1.0 g fw l^{-1}.

The influence of temperature and photoperiod on growth and reproduction was also assessed. Growth rates of *Porphyra dioica* were significantly affected by temperature and photoperiod. The highest growth rate, 27.5% fw day^{-1}, was recorded at 15ºC and $16:\overline{8}$ h, $L:\overline{D}$ cycle. Formation of young bladelets in the basal portion of unfertilized female thalli was observed after 4 weeks in culture. After 7 weeks all biomass produced was solely due to these vegetatively propagated young thalli, growing 22.4 to 26.1% day^{-1}.

The species ability to uptake and accumulate high quantities of N was assessed for increasing concentrations of two forms, nitrate (NO_3^-) and ammonium (NH_4^+). The growth rate of *Porphyra dioica* increased linearly with the increase of the N concentration up to 500 µM NO_3^-. *Porphyra dioica* was able to grow equally well using NO_3^- or NH_4^+ as source of N and NH_4^+ was not toxic at concentrations as high as 300 µM. The results of the diel uptake experiment showed that *Porphyra dioica* prefers ammonium to nitrate when both forms are present. The amount of NH_4^+ removed during the light period was the double than during the night period. The amount of NO_3^- increased slightly during the night period and the amount of PO_4^{3-} remained constant during the 24 hours.

The results obtained during this work showed that *Porphyra dioica* is a good candidate for application in integrated aquaculture systems. Evidences for this are: high growth rates, achieved over a range of temperatures and photoperiods; ability to uptake and accumulate high amounts of N, under high concentrations and at least in two different forms (NO_3^- and NH_4^+); possibility of vegetative propagation of the blades. Application of this species will depend on future studies on large scale systems and assessment of the quality/value of its biomass.

RÉSUMÉ

Le genre *Porphyra* est l'un des genres le plus importants de macroalgues marines utilisés dans l'aquaculture, tant en valeur monétaire qu'en biomasse produite. Selon les donnés de la FAO (Organisation des Nations Unies), en 2001, 1,132 millions de tonnes de *Porphyra* on été produites, évaluées en 1,2 billions de dollars. Cette valeur de production représente 16,3% du total d'aquaculture de macroalgues, qui à la fois, correspond à 28,4% de la production totale mondiale d'aquaculture (y comprís des poissons, mollusques et crustacés. Les algues du genre Porphyra sont surtout utilisées dans l'alimentation humaine et comme source du pigment *r*-ficoeritrine.

Cette étude a été exécutée de façon à augmenter la connaissance sur la biologie de *Porphyra dioica*, unes des espèces de *Porphyra* les plus communes dans la côte nord du Portugal. Le premier but était de caractériser le cycle de vie de cette espèce dans la nature et en laboratoire. La surveillance du pourcentage de couverture de populations naturelles a montrée que *P. dioica* existe pendant toute l'année dans cette zone. Les mêmes études ont révélée un plus grand pourcentage de couverture, 23 et 66%, entre Février et Mai. De façon à caractériser le cycle de vie en laboratoire, ont été testés les effets de la température, intensité lumineuse et photopériode dans la germination des zigotospores et dans le développement et la reproduction de la phase conchocelis. Les zigotospores germent plus rapidement à 15ºC et avec 25 à 75 µmol photon m^{-2} s^{-1}. La taux de croissance des conchocelis a été influencé par la température plus que par le photopériode et l'intensité lumineuse. Dans les trois photopériodes testés le taux de croissance a été toujours plus élevée à 15ºC et avec 25 à 75 µmol photon m^{-2} s^{-1}. La fréquence de la formation de conchosporangios à été plus élevée à 15ºC avec 8 heurs de lumière pour jour et 25 à 75 µmol photon m^{-2} s^{-1}, étant presque inexistante en les cultures de conchocelis en suspension. Les conditions excellentes pour la formation de conchosporangios se sont aussi révélées les meilleures pour la libération de conchospores après 18 semaines. L'aérage des cultures est une condition essentielle pour la germination des spores et l'obtention de gamétophytes. On n'a pas observé la formation de arqueospores d'aucun autre type de spores asexués.

Le deuxième objectif principal était de déterminer les conditions excellentes pour la croissance des gamétophytes et vérifier son potentiel pour l'usage en aquaculture intégrée pour absorption des éléments nutritifs. En ce sens à été testé l'influence de la densité de culture (pf – poids frais) et l'intensité lumineuse dans la taux

de croissance, productivité et absorption des éléments nutritifs. Le taux de croissance maximum, 33% par jour, a été enregistré en des cultures avec 0,1 g pf l^{-1}. Le taux de croissance a diminué de façon significative avec l'augmentation de la densité de la culture, en même que la productivité a montré une tendance inverse. À 150 µmol photon m^{-2} s^{-1} et avec 1,5 g pf l^{-1} on été produit 1,4g pf par semaine. Avec cette intensité lumineuse on n'a pas enregistré une différence significative dans la productivité à des densités entre 0,6 et 1,5 g pf l^{-1}. Le conteneur d'azote (N) des algues a diminué avec l'augmentation de la densité de culture et de l'intensité lumineuse. Le plus grand remuement de N, 1,67 mg N $jour^{-1}$, à été enregistré dans les cultures avec 1,5 g pf l^{-1} et recevant 150 µmol photon m^{-2} s^{-1}. Cependant, le N remuée par les algues à 50 µmol photon m^{-2} s^{-1} à été statistiquement égal à celui qui à été remuée par les cultures à 150 et 250 µmol photon m^{-2} s^{-1}, à la densité de 1,0 g pf l^{-1}.

L'influence de la température et du photopériode dans la croissance et la reproduction des gamétophytes a été aussi testée. Les taux de croissance de *Porphyra dioica* ont été statistiquement affectés par les deux facteurs. Le taux de croissance le plus élevé, 27,5% pf jour-*1*, a été observé à 15ºC et 16h lumière pour jour. La formation des jeunes gamétophytes dans la base des gamétophytes féminins pas fécondés a été observée après 4 semaines en culture. Après 7 semaines la biomasse produite était le résultat de la croissance de ces nouvelles lames, avec les taux de croissance entre 22,4 et 26,1% jour-1.

La capacité de croître et d'assimiler N en grandes quantités a été testée par les concentrations croissantes de N sous deux formes, nitrate (NO_3^-) et ammonium (NH_4^+). Les taux de croissance de *Porphyra dioica* ont augmenté de façon linéaire avec l'augmentation de la concentration de N dans le milieu, au moins jusqu'à 500 µM NO_3^-. *Porphyra dioica* a grandit également bien en utilisant NO_3^- ou NH_4^+ comme source de N et on n'a pas observé des effets toxiques du NH_4^+ au moins dans les concentrations testées. Quant aux cycles quotidiens de remuement de éléments nutritifs, le NH_4^+ remué pendant la période de lumière a été le double de celui qu'on à remué pendant la nuit. Le remuement de NO_3^- a augmenté légèrement pendant la nuit et la remuent de phosphate est resté constant. Dans cette expérience on a constaté encore qu'en présence de deux sources de N, *P. dioica* utilise préférablement le NH_4^+.

Les résultats obtenus dans ces travaux ont montré que *Porphyra dioica* est une candidate à l'application en aquaculture intégrée. Les évidences pour ce fait sont: 1) ses hauts taux de croissance, obtenus dans une considérable amplitude de températures et

photopériodes ; 2) la capacité de remuer et accumuler grandes quantités de N, sous concentrations élevées et sur au moins deux formes (NO_3^- et NH_4^+) ; 3) la possibilité de propagation végétative des gamétophytes. L'application de cette espèce va dépendre des futures travaux en des systèmes en plus grande échelle et de l'étalonnage de la qualité et valeur de sa biomasse.

ABREVIATIONS

ATP	adenosine triphosphate
B	boron
C	carbon
Ca	calcium
CA	enzyme carbonic anhydrase
Cl	chlorine
Co	cobalt
CO_2	carbon dioxide
Cu	copper
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, which inhibits the action of an anion exchange protein
dw	dry weight
FAA	free amino acids
FAO	food and agriculture organisation of the United Nations
Fe	iron
fw	fresh weight
GTP	guanine triphosphate
H	hydrogen
HCO_3^-	bicarbonate
I	iodine
K	potassium
LD	long day (in this study the cycle was 16h light and 8h dark)
MAA	micosporine like amino acids
Mg	magnesium
Mn	manganese
Mo	molybdenum
N	nitrogen
Na	sodium
ND	neutral day (12 hours light and 12 hours dark per day)
NH_4^+	ammonium
NO_2^-	nitrite
NO_3^-	nitrate
O	oxygen
P	phosphorus
PAR	photosynthetically active radiation (400-700 nm)
PBP	phycobiliproteins

PC	phycocyanin
PE	phycoerythrin
PFD	photon flux density (always in (µmol photons m^{-2} s^{-1})
PO_4^{3-}	phosphate
PSII	photosystem II
rbcL	ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit gene
S	sulphur
SD	short day (in this study the cycle was usually 8h light : 16h dark, except for chapter 3 where a 10:14h cycle was used in one experiment, see respective methods)
Se	selenium
SEAPURA	Species Diversification and Improvement of Aquatic Production in Seaweeds Purifying Effluents from Integrated Fish Farms. (www.seapura.com)
Si	silicon
SSU	small subunit ribosomal RNA gene
UVB	ultraviolet B radiation (280-320 nm)
V	vanadium
VSE	Von Stosch enriched seawater
Zn	zinc

INDEX

CHAPTER 1

INTRODUCTION

INTRODUCTION

The genus *Porphyra*, or nori (its Japanese name often used in commerce), is one of the most commercially important seaweed in aquaculture. According to the Food and Agriculture Organization of the United Nations, in 2001, 1.132 million metric tons of *Porphyra* were produced which was valued at 1.2 billion USD (FAO, 2003). The production of *Porphyra* represents 16.3% of the world's seaweed mariculture which in turn corresponds to 28.4% of the world's total mariculture production including fish, aquatic plants, molluscs and crustaceans. *Porphyra* is mainly used for human consumption as an ingredient of the Japanese delicacy "sushi". It has a high protein content (25-50% dry weight), as well as vitamins, minerals and fibres (Noda, 1993). This alga, although not traditionally included in the diet of the western world, it is increasing in popularity in vegetarian and macrobiotic diets, as a simple search on the internet can show. The taurine and *r*-phycoerythrin contents of this seaweed are also significant. Taurine is a substance that controls blood cholesterol (Tsujii *et al.*, 1983). The red pigment r-phycoerythrin is used as fluorescent marker in *in situ* hybridization studies (Mumford & Miura, 1998). *Porphyra* has other biologically active substances beneficial for human health. For instance, Zhang *et al.* (2003) showed that a sulphated polysaccharide fraction from *P. haitanensis* can be used to compensate the decline in total antioxidant capacity and the activities of antioxidant enzymes. This suggests it may be helpful in retarding the aging process. Other works with polysaccharides extracted from *P. yezoensis* showed anticoagulant (Zhou *et al.*, 1990) and immune-stimulating activities (Yashizawa *et al.*, 1995). Saito *et al.* (2002) showed that *Porphyra* peptides induced a significant reduction of the blood pressure in hypertensive patients.

After unsuccessful attempts to cultivate Japanese strains in North America, research efforts are now focused on the domestication of local species and evaluation of its potential value for aquaculture and bioremediation (Yarish *et al.*, 1998). As Stekoll *et al.* (1999) emphasised, environmental responses may be species and strain specific. Consequently, a selection process for the better *Porphyra* species and strains for aquaculture application is required (Chopin *et al.*, 1999). Such approach is already ongoing by Yarish *et al.* (1999) for species from the coast of northeast United States. There are not any similar projects going on in Europe.

In western countries, the interest for integrated aquaculture began toward the end of the 20th century. After the initial work by Ryther *et al.* (1975), interest in using algae as nutrient scrubbers in integrated aquaculture was renewed by Fujita *et al.* (1989), Kautsky and Folke (1991), Neori *et al.* (1991), Krom *et al.* (1995), Buschmann (1996), Sphigel and Neori (1996), Troell *et al.* (1997), Chopin and Yarish (1998), Neori and Shpigel (1999) and Yarish *et al.* (1999), among others. Recently, the European Union sponsored the project SEAPURA - Species diversification and improvement of aquatic production in seaweeds purifying effluents from integrated fish farms - aiming the development and test of the cultivation of high-value seaweed species (marine macroalgae) not used before in poly-aquaculture. With integrated aquaculture practices, the wastes of the animal culture are used by the algae to grow, resulting in an added production of valuable biomass. At the same time there is a substantial reduction of the load of inorganic nutrients in the effluents from intensive aquaculture, that constitutes a potential ecological problem.

The loading of inorganic nutrients in some coastal waters has been the consequence of the increasing finfish aquaculture (Ackefors & Enell 1990, 1994). When this load exceeds the carrying capacity of the environment severe disturbances can

occur, including diseases, eutrophication, harmful algal blooms and green tides (Folke & Kautsky, 1989). It is worth noting, however, that aquaculture is not the only industry responsible for eutrophication of coastal waters. Galloway *et al.* (1995) calculated that the 90-130 million tons of N fixed per year before the 19th century (essentially due to biological N_2 fixation) had about doubled as of 1990. Main causes for this increase are the production of synthetic fertilizers, intensive agricultural crops atmospheric deposition associated with fossil fuel combustion and most significantly the increase in human populations. However, aquaculture's contribution to the environmental problems mentioned above should not be neglected. Data available from the European Environmental Agency (Anonymous, 1999) refers that 3 to 8 thousand tons of phosphorous and 30 to 60 thousand tons of nitrogen are currently released per year by European aquacultures. Besides, aquaculture farms maybe identified as point sources of nutrients and thus allow a direct intervention for prevention or bioremediation (McVey *et al.*, 2002).

Integrated mariculture has been practiced traditionally in China, Japan and South Korea, where farms of fish net pens, shellfish and seaweed have been placed close to each other (Rawson *et al.*, 2002; Neori *et al.*, 2004). Optimal integration has been achieved through trial and error, but the information for quantification and design has seldom been published (*e.g.* Fang *et al.*, 1996; Sohn, 1996; Fei, 2004). In these countries macroalgae are considered nutrient removers. For instance, the production of *Laminaria japonica* (Japanese kelp) was estimated at near 4.6 million tonnes in 2001 (FAO, 2003). Considering an N content of 2.79% dw and a wet to dry ratio of 5:1 (Egan and Yarish, 1990), we can estimate that 5.4 kg of N will be removed from the water with every ton of *Laminaria* produced. Production of *Porphyra* is lower but its N content can go above 7% (Chopin *et al.*, 1999).

On a global scale, mariculture of extractive organisms (seaweeds and shellfish) already removes a significant fraction of nutrients from the oceans (McVey *et al.*, 2002). According to Troell *et al.* (2003), the harvests of those organism already extract roughly 150 000 metric tons of N. However, as those authors also note, extractive and fed aquaculture are very often geographically apart, rarely balancing each other at the regional scale. An environmentally sustained integrated aquaculture operation recreates a mini-ecossystem. In those systems, the plant autotrophy balances the animal and microbial heterotrophy, not only in terms of nutrient removal (particularly C, N and P) but also with respect to oxygen, pH and carbon dioxide (Rai *et al.*, 2000; McVey et al., 2002).

Due to its morphological characteristics, high surface area to volume ratio (SA/V), *Porphyra* is considered a good candidate for integrated aquaculture application. The thallus of *Porphyra* is a thin blade with one or two layers of cells, all potentially involved in nutrient absorption. It can be argued that a thallus with high surface area to volume ratio does not allow storage of nutrients in reserve tissues like those of brown algae (*i.e.* Laminariales and Fucales). The advantage of *Porphyra* is its rapid growth, up to 25% per day (Chopin *et al.* 1999; Pereira *et al.* submitted**;** Carmona *et al.* submitted), which can allow repeated harvest and continuous removal of nutrients from the water.

The best growth rates and nutrient removal capacities are usually found in species with high surface area to volume ratios as explained by the functional-form model (Hanisak *et al.*, 1990; Littler & Littler, 1980). For that reason, a lot of work has been done using thin blade-like species of Chlorophyta, like *Ulva* and *Enteromorpha*. Martínez-Aragón *et al.* (2002) compared phosphate removal, from sea bass cultivation effluents, by *Ulva rotundata*, *Enteromorpha intestinalis* and *Gracilaria gracilis*. The maximum P uptake rate (2.86 µmol PO_4^{3+} g^{-1} dw $hour^{-1}$) was found in *U. rotundata*. In a

follow up study with the same species (Hernández *et al.*, 2002), *U. rotundata* also showed the highest NH_4^+ uptake rate, 89.0 µmol g^{-1} dw $hour^{-1}$. Chung *et al.* (2002) recorded an uptake rate of 114.6 µmol NH_4^+ g^{-1} dw $hour^{-1}$ for *U. pertusa*. Harlin (1978) reported an uptake rate of 129.4 µmol NO_3^- g^{-1} $hour^{-1}$, for *Enteromorpha* sp.. Fujita (1985) obtained, for *U. lactuca*, uptake rates between $2x10^3$ and $3.6x10^3$ µg N g^{-1} dw $hour^{-1}$. The same author obtained, for *E. intestinalis*, a maximal uptake of $14x10^3$ µg N g^{-1} dw $hour^{-1}$.

Initial studies with *Porphyra* showed that some species of this genus perform well in terms of nutrient removal (Chopin *et al.*, 1999; Kraemer and Yarish 1999; Carmona *et al.*, submitted). Chopin *et al.* (1999) showed that the phosphate (P) and nitrogen (N) in the tissue increased in *Porphyra* specimens growing near salmon cages. Of the several species studied, those authors concluded that *Porphyra yezoensis* and *Porphyra purpurea* respond to high nutrient loading in coastal waters resulting from anthropogenic activities (salmon aquaculture and intense scallop dragging). Tissue P and N for those *Porphyra* species reached 8.5 mg P g^{-1} dw and 73.9 mg N g^{-1} dw. These values are higher than the ones recorded for species like *Ascophyllum nodosum*, 3.2 mg P g^{-1} dw, *Polysiphonia lanosa*, 3.7 mg P g^{-1} dw and *Pilayella littoralis*, 5.4 mg P g^{-1} dw (Chopin *et al.*, 1996). Gallant *et al.* (1993) found a maximum of 5.2 mg P g^{-1} dw in *Chondrus crispus*. As for N content, the highest value recorded was 26.8 mg N g^{-1} dw in *A. nodosum*, 43.0 mg N g^{-1} dw in *P. lanosa*, 41.4 mg N g^{-1} dw in *P. littoralis* (Chopin *et al.*, 1996) and 45.1 mg N g^{-1} dw in *C. crispus* (Gallant, 1993).

Studies in the laboratory have also shown that *Porphyra* responds to high concentrations of NH_4^+, a common form of N in water enriched by anthropogenic activities. Wu *et al.* (1984) studied the utilization of ammonium (NH_4^+) by *Porphyra yezoensis* and obtained higher growth rates (11.6% day^{-1}) and tissue nitrogen content

(4.72% dw) with NH_4^+ concentrations of 5 to 10 ppm (≃350 to 700 μM). For the same species, Amano and Noda (1987) concluded that the optimal fertilizing effect was obtained using 20 ppm N as NH_4^+ for 48h cultivation. Recently, Carmona *et al.* (submitted) assessed the bioremediation potential of several native northeast American species of *Porphyra* and compared those to well-known Asian species. In that study, growth and tissue N reached maximal levels at inorganic N concentrations of 150-300 μM. Maximum growth rates ranged from 10 to 25% day^{-1}.

Due to the present and the above mentioned studies, *Porphyra* is now being considered as an important candidate for application in integrated aquaculture and bioremediation. Besides, the N content of *Porphyra* is higher than that of other commonly cultivated seaweeds like *Laminaria* and *Gracilaria* (Table 1). Species of these genera, with actual application and commercial value, are being considered to help solving coastal eutrophication in China (Fei, 2004). As Fei (2004) points out, whatever species is used for integrated aquaculture and bioremediation, besides all other requisites, there should be a market for it.

Table 1: Nutrient contents (% dw) of cultivated seaweed.

Contents	*Laminaria*	*Porphyra*	*Gracilaria*
Protein	8.20	39.00	16.30
Carbohydrates	62.70	43.00	78.80
C	23.60	27.30	31.30
N	1.30 2.79*	6.20	2.60
P	0.20	0.58	0.03

Adapted from Fei (2004). * Data from Egan and Yarish (1990) for *Laminaria longicruris*.

TAXONOMIC POSITION AND MORPHOLOGICAL CHARACTERIZATION

The genus *Porphyra* belongs to the division Rhodophyta, order Bangiales, family Bangiaceae. Unlike *Bangia*, the other genus of this family, *Porphyra* consists of relatively large sheets of cells. Nevertheless, Muller (2000) proposed that marine *Bangia* and *Porphyra* should be grouped in the common generic name of *Bangia* in which there are two morphotypes - *Porphyra* and *Filiformia* - until research can further clarify the status of infrageneric taxa. The same author also recommends that freshwater collections of *Bangia* should be under the new specific epithet of *Pseudobangia atropurpurea*.

Despite its simple morphology, *Porphyra* is one of the oldest red algae known. Fossils that closely resemble this genus were found in the 570 million year old Doushantuo formation in southern China (Graham & Wilcox, 2000). Presently there are at least 140 species described worldwide (Yoshida *et al.*, 1997; Silva, 1999). This number tends to increase as more taxonomic studies are carried out with more accurate molecular tools (Lindstrom and Cole, 1990; Brodie *et al.*, 1998; Broom *et al.*, 2002; Klein *et al.*, 2003).

Morphologically, the genus *Porphyra* is within the simplest red algae. Its macroscopic phase consists of thalli with either one (monostromatic) or two cells layer (distromatic). This is usually considered a species-specific character, although Brodie *et al.* (1998) proved it has no taxonomic significance. Molecular systematic studies suggest that the monostromatic type may be older and that the distromatic type may have arisen at least twice (Oliveira *et al.*, 1995).The blades of *Porphyra* grow by division of the marginal cells and attach to the substrate (rocks, shells or other algae) through numerous thin, colourless rhizoidal cells. The genus is typically divided in three

groups, based on the number of cells and on the number of chloroplasts per cell. There are monostromatic species with one chloroplast per cell, monostromatic species with two chloroplasts per cell and distromatic species with one chloroplast per cell (Bold & Wynne, 1985). Due to this simple but often variable morphology, the identification of different species of *Porphyra* is difficult. Some of the characters used are: shape, thickness and colour (in fresh and dry conditions) of the blades; distribution of the reproductive cells (monoecious or dioecious species); arrangement of the reproductive cells; shape and size of the vegetative cells; shape of the margins and of the marginal cells; seasonality of the gametophyte phase; position on shore (in relation to the tidal level); and, number and size of the chromosomes (Hus, 1902; Coll & Oliveira Filho, 1976; Wilkes *et al.* 1999).

To solve the taxonomic problems associated with morphology and phenotypic plasticity, more studies are focusing on molecular techniques, sequencing the genes of the Rubisco units or the nuclear small subunit ribosomal RNA gene (SSU) (Brodie *et al.*, 1996; Klein *et al.*, 2003). There is a need to find genes capable of distinguishing species, without detecting differences between populations of the same species, which could lead to formation of many "new" species. As Klein *et al.* (2003) pointed out, phylogenetic analysis of the Bangiophyceae have previously been conducted based on the sequences of a single or, at most, two examples of each species. This limited sampling is inadequate to assess intraspecific variation or to reveal cryptic variation in morphologically similar taxa. Those authors developed molecular screens that can reliably and objectively sort specimens of northwest Atlantic *Porphyra* into taxa (Klein *et al.*, 2003). It is a DNA-based system that uses partial sequences of the nuclear small subunit ribosomal RNA gene (SSU) or the plastid ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit gene (*rbc*L). A similar study was carried out for

Porphyra and *Bangia* species from the northeast Atlantic (Brodie *et al.*, 1998). Nevertheless, morphological and life history traits are still useful and are used to distinguish species.

LIFE CYCLE

The life cycle or *Porphyra* was described for the first time by Kathleen Drew (1949, 1954). She was the first to suggest that the microscopic, shell inhabiting, red algal "species" "*Conchocelis rosea*" Batters was, in fact, a phase in the life history of *P. umbilicalis* var. *laciniata* and not an autonomous species. This had an enormous impact in the aquaculture of *Porphyra*. Up to this date man's action was empirical and limited to provide more attachment substrate in places where *Porphyra* appeared every year. These findings by Drew enabled seaweed farmers to begin controlling the cycle and cultivation of *Porphyra* on a systematic basis.

The life cycle of *Porphyra* is biphasic and heteromorphic, with a foliose haploid gametophyte and a filamentous diploid sporophyte (figure 1). The sporophyte phase it is commonly referred as the conchocelis phase. The conchocelis serves as a perennating stage in nature and it can also be maintained in laboratory cultures for long periods, through vegetative propagation. In some species the conchocelis can also reproduce through alternative spore formation, like neutral conchospores and conchocelis archeospores (*sensu* Nelson *et al.*, 1999). The neutral conchospores are formed by differentiation of vegetative filaments. Each sporangium produces a single spore which germinates, sometimes *in situ*, to form further conchocelis. The archeospores are formed

by differentiation of a vegetative cell, which releases a single cell product that germinates to form new conchocelis (Sahoo *et al.*, 2002).

The vertical distribution of the sporophyte phase is not clear. Martinez (1990) refers that the conchocelis of *Porphyra nereocystis* inhabits the subtidal zone, just like the macroscopic stage, which is an epiphyte on *Nereocystis luetkeana* (Phaeophyta). Although it is reasonable to assume that the conchocelis exists close to the area inhabited by the blade-phase, it may live in water as deep as 78 m (Lüning, 1990). This is much deeper than the gametophytes.

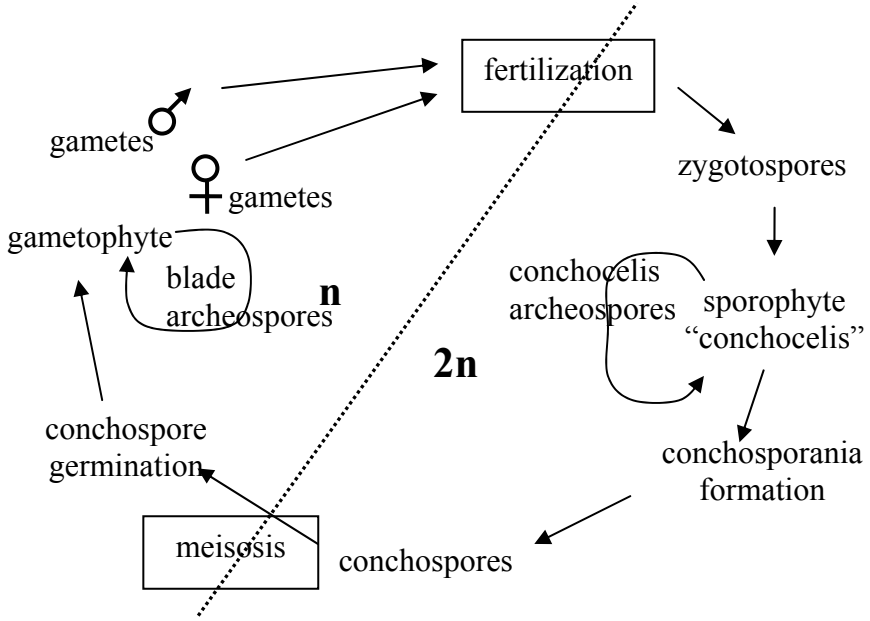

Figure 1 – Schematic representation of the life cycle of *Porphyra*.

Under appropriate environmental conditions, the conchocelis produces rows of conchosporangial filaments that produce the diploid chimeric conchospores (Mitman and Van der Meer, 1994). The conchospores that are produced in the conchosporangial branches arise from the apex or from the lateral vegetative filaments. They are usually thicker than vegetative conchocelis and sometimes are referred to as "fat" filaments.

Conchospores were once thought to be the products of meiosis, but now it is generally accepted that, at least for monoecious species, meiosis occurs during the germination of the conchospores (Mitman & van der Meer, 1994). During the germination of the conchospores, meiosis yields a four haploid celled germling, a chimera. The meiotic process segregates alleles for sexual determination and subsequent mitotic proliferation for taxa that are sectored thallus (Mitman & van der Meer, 1994). In some species this is revealed in a "patch-work" effect, as some sectors produce spermatia whereas others produce carpogonia. However, confirmation of the position of meiosis is only available for four species of *Porphyra*: *P. yezoensis* (Ma & Miura, 1984; Tseng & Sun, 1989), *P. torta* (Burzycki & Waaland, 1987), *P. tenera* (Tseng & Sun, 1989) and *P. purpurea* (Mitman & van der Meer, 1994). Burzycki and Waaland (1987) and Guiry (1991) suggest that meiosis may not be a fixed event, varying according to species or growing conditions. Further studies are needed to confirm the position of meiosis in other species of *Porphyra*, especially the dioecious species such as *P. dioica*.

Mitman and van der Meer (1994) also have reported that the mature blade of *Porphyra purpurea* is almost entirely derived from the two anticlinally divided upper cells of the original meiotic tetrad. This results in an adult blade composed of two large, side-by-side sectors. A similar observation was previously reported by Ohme *et al.* (1986) for *P. yezoensis*. In *P. yezoensis* germinating conchospores results in a linear tetrad of cells and consequently, the adult blades are horizontally divided instead of vertically divided as in *P. purpurea*.

The distribution of the reproductive cells varies between species and can be used as a distinctive character. There are monoecious species, where spermatia and carpogonia coexist in the same individual, and dioecious species, when the gametes are formed in distinct individuals (female blades and male blades). Within the monoecious

species there is also different distribution of the reproductive cells. In some species the blade presents a longitudinal division, with male regions on one side and female regions on the other, i.e., sectored. In other species there are patches of male and female cells throughout the blade in no particular order. In *Porphyra dioica*, a dioecious species, Holmes and Brodie (2004) report the occurrence of individuals with male and female reproductive structures on the same blade.

Formation of archeospores (*sensu* Nelson *et al.*, 1999) can also occur in the gametophyte phase. In this case the released spore germinates into a new blade. These spores were previously referred to as monospores. This term is no longer considered appropriate and is reserved for use when single spores are produced by an unequal cell division, according to Magne (1991). Still researchers seem to still misuse this term. The difference is in the origin of the spores - blade archeospores or monospores and conchocelis archeospores or monospores - since the results of its germination are the same, both form new blades and new conchocelis, respectively. In both cases the formation of acheospores is described only for some species, while others do not present any kind of asexual spores. Besides archeospores, other kinds of asexual spores (agamospores, neutral spores, endospores) have been described in *Porphyra*. Nelson *et al.* (1999) reviewed the terminology used to describe reproduction and life history stages in *Porphyra*.

A list of species of *Porphyra* with asexual reproductive modes was published by Notoya (1997) in his paper on diversity of life history in the genus. In that study, the author recognizes four types of life histories of the genus *Porphyra*. In the *Porphyra tenuipedalis* type (type I), spermatangial and carpogonial patches are mixed on the foliose thallus. After fertilization, zygotospores are formed, liberated and germinate to form the conchocelis. Spherical cells are formed at the tips of the conchocelis filaments

and develop directly into new foliose thalli. Production of asexual spores is not known in species of this type. In the *Porphyra lacerata* type (type II), spermatangial and carpogonial patches are also mixed on the foliose thalli. After fertilization, the zygotospores are released and germinate to produce conchocelis. The conchocelis produces conchosporangial branches where the conchospores will be formed. After being released, the conchospores germinate into the foliose phase. Asexual reproduction modes have been described for some species of this type. In the *Porphyra variegata* type (type III) the thalli are also monoecious, but with male and female tissue separated on longitudinal halves. Asexual spore formation is known in the foliose and in the conchocelis phase. Finally, in the *Porphyra dentata* type (type IV), spermatia and carpogonia are usually produced on different thalli (dioecious species), although some monoecious thalli can be found. When monoecious, the thalli are sectored by an almost horizontal line. Asexual spores from the foliose thallus have been reported for some species of this type.

Typically, each phase of the life cycle has specific environmental requirements: temperature, photoperiod, and photon flux density (PFD). Other factors that can influence reproduction are light quality, nutrient availability, salinity and the kind of substrate (for conchocelis). In some species, the requirements for reproduction are very strict. For instance, the conchosporangia of *Porphyra linearis* releases spores only at 13ºC (Bird, 1972). *Porphyra miniata* forms conchosporangia only at 13-15ºC and low light (ca. 3.6-7.2 µmol photons m^{-2} s^{-1}) from $10:\overline{14}$ to $16:\bar{8}$ h L:$\overline{D}$ cycles. The release of spores occurs at lower temperatures (3-7ºC), higher light intensity (ca. 5.4-27.2 µmol photons m^{-2} s^{-1}) and only with $8:\overline{16}$ h L:$\overline{D}$ cycle (Chen *et al.*, 1970). Many species, *e.g.* *Porphyra miniata*, *P. torta*, *P. linearis* and *P. columbina* are seasonal and thus controlled primarily by photoperiod (Chen *et al.*, 1970; Bird *et al.*, 1972; Avila *et al.*,

1986; Waaland *et al.*, 1987). In other species, like *Porphyra moriensis*, the conchocelis survives at 5-25ºC with good growth rates at 15-25ºC, PFD of 20-80 µmol photons m^{-2} s^{-1}, under $10:\overline{14}$ and $14:\overline{10}$h L:$\overline{D}$ cycles (Notoya & Miyashita, 1999). The foliose thallus also develops under various temperatures and both photoperiods mentioned.

Each species is obviously adapted to the environmental conditions of the region were it exists. This eliminates any chances of generalization of the optimal culture conditions from already studied species. On the other hand, species from the same region can have different requirements according to its seasonal occurrence. There are, however, other factors that influence algae in the natural habitat and that can prevent the expected occurrence of the algae based solely on laboratory data. Factors like competition with other species and herbivores and the kind of substrate available are known to influence the distribution of species in nature (*e.g.* Terawaki *et al.*, 2001). These factors should also be taken into account when inferring about the species' ecology from laboratory results.

Porphyra dioica Brodie *et* Irvine is one of at least 5 species described in Portugal (Ardré, 1970**;** South & Titley, 1986**;** Brodie & Irvine, 1997) and the most common in the North of Portugal together with *Porphyra umbilicalis* (L.) Kützing. *Porphyra dioica* specimens are ephemeral, but can be found during the entire year in the intertidal zone, on rocks. This species was described in 1997, being separated from the already known *Porphyra purpurea* (Roth) C. Ag. (Brodie and Irvine, 1997). For that reason its distribution is not well known. The blades are lanceolate to broadly ovate with up to 270 mm long and 270 mm broad. Sometimes the blades are laciniate and with ruffled margins. The blade colour varies from olive-green to purple-brown. The blades are monostromatic, 48 to 80 µm thick. The sexual areas are in the margins and usually in upper two thirds of the blade. As indicated by its name, this species is

dioecious. The female areas have a brick-red colour and zygotospores are formed in packs of 8 with a 2x2x2 arrangement. The male areas are pale-yellow with spermatia in packs of 64 with a 2x4x8 arrangement (Holmes and Brodie, 2004, Pereira *et al.*, accepted).

FACTORS THAT CONTROL SEAWEED PRODUCTIVITY

Temperature

The surface temperatures of the oceans vary in two ways: they decrease from about 28ºC in the tropics to 0ºC towards the poles; and they have seasonal changes that are larger in mid-latitudes than in the tropics or the poles (Lüning, 1990). In the intertidal zones the temperature regimes are much more complex than in subtidal zones. The way the temperature affects intertidal species during tidal cycles varies, in part, with the morphology of the plant. The temperature at the interior of a clump of *Endocladia muricata*, a stiff, tufty plant, remains lower than the air or the open rock surface temperature, during low tide. *Porphyra fucicola*, on the other hand, is flattened against the rock surface and becomes much hotter than air (Biebl, 1970 as cited in Lobban and Harrison, 1994).

Temperatures have fundamental effects on chemical reactions and, consequently, on metabolic pathways (Lobban & Harrison, 1994). Several studies have

investigated the influence of temperature on growth and photosynthesis (*e.g.* Breeman *et al.*, 2002; Orfanidis, 2001; Orfanidis and Breeman, 1999; Gordon *et al.*, 1980). Examples of the influence of temperature on the reproduction of *Porphyra* species were mentioned in the life cycle section. For instance, the conchosporangia of *Porphyra linearis* releases spores only at 13ºC (Bird, 1972**)**. *Porphyra miniata* forms conchosporangia only at 13-15ºC and releases conchospores only at 3-7ºC in combination with light and photoperiod conditions (Chen *et al.*, 1970). In *Porphyra columbina* the release of conchospores requires a decrease in temperature from 15 to 10ºC (Avila *et al.*, 1986).

Temperature optimum varies among species and among strains, as well as between the different phases of heteromorphic life histories. For instance, in *Porphyra dentata* and *Porphyra pseudolinearis*, fastest growth of the conchocelis was observed at 20ºC for both species, whereas the foliose thalli grew better at 10-15ºC and 5ºC, for each species (Nam-Gil, 1999). There are also differences in temperature optima with the development stage of the thallus. For example, the optimum temperature for cultivation of *Porphyra yezoensis* drops from 20ºC at the time of conchospore germination to 14-18ºC for thalli with 1-2 cm long and even lower for larger thalli (Tseng, 1981). In *P. pseudolinearis*, foliose thalli up to 20 mm long, grows faster at 15ºC whereas after that size the thalli grows faster and bigger at 5ºC (Nam-Gil, 1999).

Ultimately, the influence of temperature in all the factors mentioned before contributes to the establishment of the species geographic distribution. In the red alga, the hypothesis of temperature boundaries based on ocean isotherms was tested by Yarish *et al.* (1984). In that study the temperature tolerance of several species was tested in combination with light intensity and day length. Those authors concluded that the

geographical boundaries of those species could be explained using the mean surface seawater isotherms.

Light

Seaweeds grow in habitats with exceptionally diverse and dynamic lighting regimes. The primary importance of light for the seaweeds is to provide energy for photosynthesis. Intertidal species must cope with both photosynthetically active radiation (PAR) and ultraviolet radiation, particularly ultraviolet B (UVB) of wave lengths 280-320 nm. The UVB radiation may damage DNA and cellular proteins like the D1 protein essential to photosystem II (PSII) in chloroplasts (Graham & Wilcox, 2000). In the field, high light stress causes dynamic photoinhibition (Hanelt, 1996). That phenomenon results in a decrease of the photosynthetic activity, by inactivation of the PSII, when incident light exceeds the electron transport capacity. In extreme cases, strong UV radiation causes photo-oxidation of the photosynthetic pigments (Häder & Worrest, 1991). In that case photoinhibition is not a reversible process.

The effects of solar radiation on photoinhibition and pigmentation have been studied for a few species of *Porphyra*. In *Porphyra leucostica*, photosynthetic efficiency and maximal photosynthesis were drastically reduced by UVB radiation when the algae were transferred from the laboratory to solar radiation for 3h (Figueroa *et al.*, 1997). Photosynthesis in *P. umbilicalis* is also affected by solar radiation (Häder *et al.*, 1999). However, UV radiation caused a much slower decrease in the photosynthetic yield of *P. umbilicalis* when compared to the results found in other algae, like *Delesseria sanguinea* and *Plocamium cartilagineum* (Dring *et al.*, 1996 as cited by Häder and

Figueroa, 1997). These results indicate that *P. umbilicalis* is well adapted to its habitat in the upper intertidal zone.

Light is also an important signal that controls seaweed growth, reproduction and synthesis of compounds like Chl *a*. Sensor pigments, including a phytochrome-like protein (Lopez-Figueroa *et al.*, 1989) and a phytochrome-like blue light photoreceptor (Lopez-Figueroa and Niell, 1989) have been proposed for several seaweeds, including *Porphyra*. These pigments are involved in photoperiodism (growth or reproduction controlled by day length) and photomorphogenesis (dependence upon a particular portion of the spectrum). Figueroa *et al.* (1995a) described the effects of blue and red light on the growth and photosynthetic metabolism in *Porphyra umbilicalis*. Growth rate was higher in red light, while concentrations of photosynthetic pigments were higher in thalli grown in blue light. Also in *Porphyra umbilicalis*, a free running rhythm was observed in constant green or red light at irradiances of 2.5-20 μmol photons m^{-2} s^{-1}, whereas arhythmicity occurred in constant blue light at 6-20 μmol photons m^{-2} s^{-1} (Lüning, 2001). According to the same work, the photoreceptors that control growth rhythmicity in *Porphyra umbilicalis* have high sensitivity for blue light and lower sensitivity for red light. Salles *et al.* (1996) suggested that the red/far-red ratio acts as a photomorphogenic signal controlling growth rate and thallus thickness in *Porphyra leucosticta*. In the same species, Figueroa (1996) showed the effects of light quality on nitrate reductase and glutamine synthetase activities (key enzymes in the inorganic N metabolism). Red or blue light pulses (5 or 15 min.) stimulated nitrate reductase activity and soluble protein synthesis in darkness. The activities of both enzymes were higher after blue than after red light pulses.

Photoperiod

Photoperiod influences growth and reproduction differently according to the species. This is true not just for *Porphyra* species but for others as well. For instance sorus formation in the sporophyte of *Laminaria saccharina* and new frond formation in *Laminaria hyperborea* requires short-day (SD) cycles (Lüning, 1986, 1988).

Porphyra leucosticta produced zygotospores and spermatia independently of photoperiod at 15ºC, but only under long-day (LD) conditions when at 5, 10 and 20ºC (Orfanidis, 2001). On the other hand, blade archeospores were observed only under SD conditions at 15 and 20ºC. *Porphyra lacerata* and *P. moriensis* are other examples of *Porphyra* species insensitive to photoperiod (Notoya and Nagaura, 1998; Notoya and Miyashita, 1999). *Porphyra torta*, *P. spiralis* var. *amplifolia*, *P. abbottae*, *P. nereocystis*, *P. perforata* and *P. columbina* are a few examples of *Porphyra* species where photoperiodic control occurs at least in one of the life history phases. In *P. torta,* the conchospores maturation and release occurs only when the cultures are exposed to a SD photoperiod (Waaland *et al.*, 1987). In *P. spiralis* var. *amplifolia* the conchospores formation and release occurs only in a day length of 12 hours or less (Kapraun & Lemus, 1987). In *P. abbottae*, *P. nereocystis* and *P. perforata*, the conchospores formation and release occur only in LD (Waaland *et al.*, 1990). In *P. columbina,* the blades form spermatia and zygotospores only in long days, while the conchospores are formed only in 12 hours-length days or less (Avila *et al.*, 1986).

In conclusion, photoperiod is the signal that triggers reproduction in many seaweed, while others are insensitive to it. In fact, photoperiod is not the only signal for reproduction. Factors like light intensity and temperature, for instance, proved to be also important, as shown by the examples referred in the life cycle section.

Nutrients

Besides carbon (C), water and light, seaweeds require various chemical elements for photosynthesis and growth. Hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), magnesium (Mg), copper (Cu), manganese (Mn), zinc (Z) and molybdenum (Mo) are considered to be required by all algae (Lobban and Harrison, 1994). On the other hand, elements like sulphur (S), potassium (K) and calcium (Ca) are required by all algae, but can be partially replaced by other elements, while sodium (Na), cobalt (Co), vanadium (V), selenium (Se), silicon (Si), chlorine (Cl), boron (B) and iodine (I) are required only by some algae (DeBoer, 1981).

The concentrations of various elements in seawater vary tremendously (Table 2). Elements present in the nanomolar range are considered micronutrients (*e.g.* Fe, Cu, Mn, Zn) for nutritional purposes. Elements occurring at higher concentrations are referred as macronutrients (*e.g.* C, N, P). Some seaweeds require trace amounts of organic-carbon compounds, although these compounds are not used as carbon sources for algal growth. This type of nutrition is named auxotrophy and the organic compounds are vitamins (Lobban and Harrison, 1994). The three vitamins routinely added to culture media are cyanocobalamin (B_{12}), thiamine and biotin. Phytoplankton requires the same vitamins as seaweeds, while most higher plants synthesize their own vitamins and do not depend on environmental sources. Examples of compounds in which the various elements are present and their probable functions in seaweeds are listed in Table 3.

The Liebig's law of the minimum, established for vascular plants, also applies to seaweeds. According to that theory, "growth of a plant is dependent on the minimum amount of foodstuff presented". In other words, the nutrient available in the smallest quantity with respect to the requirements of the plant will limit its growth rate, if all

other factors are optimal. Nitrogen, P, Fe, Cu, Zn, Mn and C might limit algal growth. Evidences for this come from the fact that the concentrations of these elements in seawater vary considerably (because of biological activity) and the concentrations of these elements in tissues are 10^4 to 10^5 greater than their concentrations in seawater (Table 2).

Table 2- Concentrations of some essential elements in seawater and in seaweeds. Adapted from DeBoer, 1981.

	Mean concentration in seawater		Concentration in dry matter		
Element	(mmol kg^{-1})	(µg g^{-1})	Mean (µg g^{-1})	Range (µg g^{-1})	Ratio of concentration in seawater to concentration in tissue
Macronutrients					
H	105000	10500	49500	22000-72000	2.1
Mg	53.2	1293	7300	1900-66000	$1.8x10^{-1}$
S	28.2	904	19400	4500-8200	$4.7x10^{-2}$
K	10.2	399	41100	30000-82000	$1.0x10^{-2}$
Ca	10.3	413	14300	2000-360000	$2.9x10^{-2}$
C	2.3	27.6	274000	140000-460000	$1.0x10^{-4}$
B	0.42	4.5	184	15-910	$2.4x10^{-2}$
N	0.03	0.420	23000	500-65000	$2.1x10^{-5}$
P	0.002	0.071	2800	300-12000	$2.4x10^{-5}$
Micronutrients					
Zn	$6x10^{-6}$	0.0004	90	2-680	$4.4x10^{-5}$
Fe	$1x10^{-6}$	0.00006	300	90-1500	$1.0x10^{-5}$
Cu	$4x10^{-6}$	0.0002	15	0.6-80	$1.7x10^{-4}$
Mn	$0.5x10^{-6}$	0.00003	50	4-240	$2.0x10^{-5}$

Table 3 – Probable functions of the essential elements in the seaweeds and examples of compounds. Adapted from DeBoer, 1981.

Element	**Probable functions**	**Examples of compounds**
Nitrogen	Major metabolic importance in compounds	Amino acids, purines, pyrimidines, amino sugars, amines
Phosphorus	Structural, energy transfer	ATP,GTP, etc., nucleic acids, phospholipids, coenzymes (induding coenzyme A), phosphoenolpyruvate
Potassium	Osmotic regulation, pH control, protein conformation and stability	Probably occurs predominantly in the ionic form
Calcium	Structural, enzyme activation, cofactor in ion transport	Calcium alginate, calcium carbonate
Magnesium	Photosynthetic pigments, enzyme activation, cofactor in ion transport, ribosome stability	Chlorophyll
Sulfur	Active groups in enzymes and coenzymes, structural	Methionine, cystine, glutathione, agar, carrageenan, sulfolipids, coenzyme A
Iron	Active groups in porphyrin molecules and enzymes	Ferredoxin, cytochromes, nitrate reductase, nitrite reductase, catalase
Manganese	Electron transport in photosystem II, maintenance of chloroplast membrane structure	
Copper	Electron transport in photosynthesis, enzymes	Plastocyanin, amine oxidase
Zinc	Enzymes, ribosome structure(?)	Carbonic anhydrase
Molybdenum	Nitrate reduction, ion absorption	Nitrate reductase
Sodium	Enzyme activation, water balance	Nitrate reductase
Chlorine	Photosystem II, secondary metabolites	Violacene
Boron	Regulation of carbon utilization(?), ribosome structure(?)	
Cobalt	Component of vitamin B_{12}	B_{12}
Bromine Iodine	Toxicity of antibiotic compounds(?)	Wide range of halogenated compounds, especially in Rhodophyceae

Nutrients may be taken by several different mechanisms. Uncharged particles soluble in lipids and gases (*e.g.* CO_2, NH_3, O_2 and N_2) can cross the lipid bilayers of the plasma membranes through passive transport, *i.e.* diffusion (Lobban & Harrison, 1994; Harrison & Hurd, 2001). The thickness of the boundary layer of water surrounding the thallus affects the uptake rate of the ions. If the water movement around the thallus is low, the boundary layer will be thick and uptake will be limited by the rate of diffusion across this layer. Water motion is thus an important factor for nutrient uptake and consequently for seaweed growth.

Ions usually have a much lower permeability than uncharged molecules. The charge of the ion makes it difficult for the ion to penetrate a membrane that is electrically polarized and contains charged groups that either repel or attract the ions (Lobban and Harrison, 1994). Therefore, a second mechanism for nutrient uptake is the facilitated diffusion. Unlike the first mechanism, the facilitated diffusion implies the existence of carriers that bind the ion at the outer membrane surface and provide a pathway across the membrane to the inner surface. Facilitated diffusion can be saturated, works only with specific ions and its susceptible to competitive and non-competitive inhibition. The third mechanism, active transport, implies the transfer of ions across membrane against an electrochemical gradient. It is energy-dependent.

Harrison and Hurd (2001) have recently reviewed the concepts of nutrient physiology of seaweeds relevant to aquaculture. Carbon, nitrogen and phosphorus are the main nutrient elements added to seaweed aquaculture systems. Nitrogen is considered the primary limiting nutrient in marine waters (Howarth, 1988). Phosphorus is the primary limiting nutrient in lakes, reservoirs and other freshwater environments (Rabalais, 2002), although there are also reports of P limitation in marine environments (*e.g.* Krom *et al.*, 1991; Flores-Moya *et al.*, 1997). In a recent review, Rabalais (2002)

refers that data for oligotrophic oceanic waters indicate that they are also likely limited by nitrogen preferentially to phosphorus over short time scales, but that both may be limiting. Another essential element, iron, supplied mainly from atmospheric dust is also usually in short supply. The main sources of "new" nitrogen in the open ocean are nitrogen-fixing cyanobacteria (Zehr *et al.*, 2001; Rabalais, 2002). These cyanobacteria are in turn limited by aeolian iron fluxes (Berman-Frank *et al.*, 2001). Lenes *et al.* (2001) noted that the input of iron during summer, from Saharan dust, had stimulatory effects on the blooms of cyanobacteria (*Trichodesmium*) in the Gulf of Mexico. The next sections will deal individually with each of the three main nutrients in seaweed aquaculture (nitrogen, carbon and phosphorus).

Factors affecting nutrient uptake rates include light (Lapointe & Tenore, 1981; Figueroa *et al.*, 1995b), temperature (Reay *et al.*, 2001), desiccation (Thomas *et al.*, 1987) and the nutrient concentration (D'Elia & DeBoer, 1978; Haines & Wheeler, 1978). There are also biological factors that influence the rate of nutrient uptake, including SA/V ratio (Wallentius, 1984), hair formation (DeBoer & Whoriskey, 1983), the type of tissue (Gerard, 1982), age of the plants (Harrison *et al.*, 1986) and its nutritional history (D'Elia & DeBoer, 1978; Fujita, 1985). Chapters three and four of this thesis address the effects of some of those factors on the nutrient uptake, specifically: photon flux density, temperature, nutrient concentration.

Nitrogen

Nitrogen uptake and assimilation in macroalgae was reviewed by Hanisak (1983). Nitrogen is of major importance in compounds such as amino acids, nucleic

acids and pigments. The most important sources of N for seaweeds are ammonium (NH_4^+) and nitrate (NO_3^-). However, not all seaweeds can grow equally well on these two sources. Nitrite (NO_2^-) is another source of inorganic N, but it is usually present in seawater at much lower levels. There are also some examples of seaweeds that can use organic nitrogen compounds such as urea and amino acids (Iwasaki, 1967; Hanisak, 1979a).

Ammonium is usually taken up at higher rates than NO_3^- (*e.g.* D'Elia & DeBoer, 1978; Haines & Wheeler, 1978; Amano & Noda, 1987) and it is considered to be less dependent upon photosynthesis than NO_3^-. Ammonium is directly converted into amino acids via glutamine synthetase. Nitrate can be stored in the vacuoles and cytoplasm or be reduced to NO_2^- via the enzyme nitrate reductase. Nitrite is then transported to the chloroplast where it is reduced to NH_4^+ via the enzyme nitrite reductase (Harrison & Hurd, 2001).

Hanisak (1983) conceptualized three major nitrogen pools in macroalgae, which he designated as structural, physiological and storage pools (figure 2). According to the same author, the size of the pools depends on the growth rate and the internal nitrogen concentration of the organism. The structural pool is the most conservative, *i.e.*, it does not increase significantly when additional N is available. It consists of essential molecules like nucleic acids and structural proteins of membranes. This pool may be close to the N subsistence level (*sensu* Hafting, 1999), *i.e.*, the minimum tissue content at which growth occurs. The physiological pool consists of nitrogen that is important to the physiological or metabolic processes of the macroalga, like enzymes and photosynthetic pigments. Depending also on the other environmental conditions that control growth, a macroalga will grow at a certain rate that will require a certain physiological pool size. This pool size is related to the critical N level (*sensu* Hanisak *et*

al., 1979b). Any N taken up, in addition to what is necessary to sustain maximum growth, will enter the storage pool. Amino acids and phycobiliproteins are considered the most important N storage compounds iin red seaweeds.

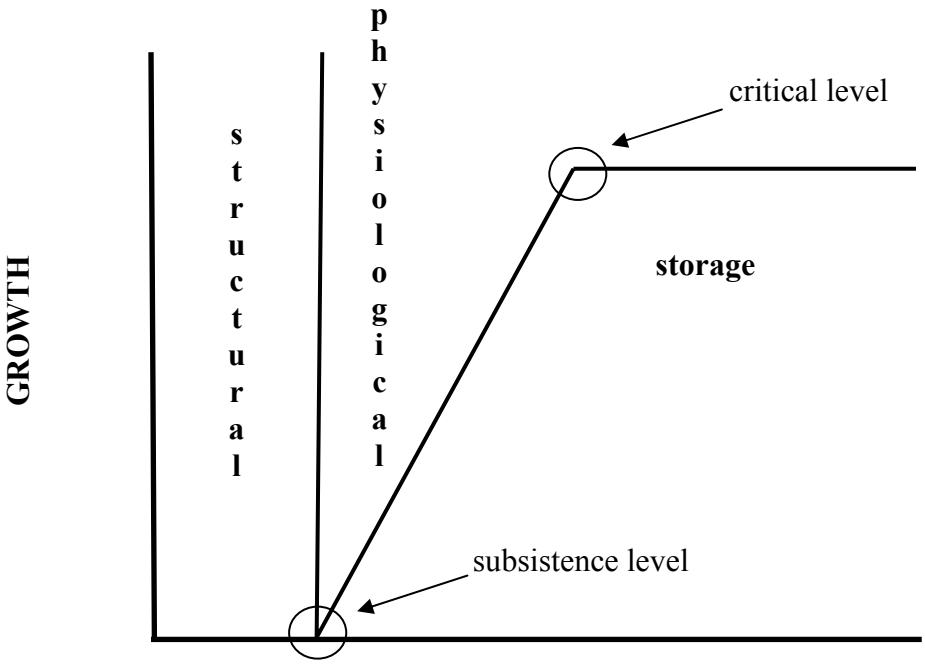

Figure 2: Schematic representation of the relationship of the growth of the macroalgae with internal nitrogen and the nitrogen pools in macroalgae. Adapted from Hanisak, 1983.

Addition of N commonly results in an increase in amino acids and photosynthetic pigments. The role of N-storage compounds varies with species and with N source. Amino acids (especially alanine) and proteins appear to be major N-storage pools in *Gracilaria tikvahiae* (Bird *et al.*, 1982). For another species of the same genus, *G. cornea*, the pigment phycoerythrin (PE) seems to be the most important N-storage compound (Navarro-Angulo & Robledo, 1999). In *Porphyra perforata*, the internal total

amino acids and soluble protein showed correlation with uptake rates of NH_4^+ and not with NO_3^- (Thomas & Harrison, 1985). Figueroa *et al.* (1995b) also detected accumulation of organic nitrogen (biliproteins, soluble proteins, amino acids) without thallus expansion in *Porphyra* sp. grown under blue light and with NO_3^- as the N source.

Inhibition of uptake of one N form due to the presence of another N form was reported for several species. The most common is the inhibition of NO_3^- uptake by the presence of NH_4^+, and the degree of inhibition is often dependent on the concentration of NH_4^+. In *Neoagardhiella baileyi* and *Gracilaria foliifera*, uptake of NO_3^- was suppressed at NH_4^+ concentrations over 5 μM (D'Elia & DeBoer, 1978). Haines and Wheeler (1978) found that NH_4^+ reduced 50% the uptake of NO_3^- in *Hypnea musciformis*, whereas in *Macrocystis pyrifera* the presence of one did not affect uptake of the other. In *Porphyra perforata*, NH_4^+ inhibits NO_3^- uptake only for the first 10-20 minutes (Thomas & Harrison, 1985).

These questions of preferential uptake of one N form over others and the inhibition of the uptake of NO_3^- in the presence of NH_4^+ are of much relevance for integrated aquaculture purposes. In an integrated aquaculture environment most of the N comes from animal excretion and is available in the form of NH_4^+. However, at this point, most intensive aquacultures use bacterial biofilters to transform NH_4^+ into NO_3^-, not toxic for the animals. In these cases, NH_4^+ is maintained at a low concentration and more N is available in the form of NO_3^-. Therefore, it is important to understand the way a species deals with these two forms of N; *i.e.* (1) which form is preferentially assimilated and (2) if there is an inhibitory effect of NH_4^+ on the uptake of NO_3^-. These questions are addressed in chapter four of this thesis.

Phosphorus

As stated before, phosphorus is not generally considered to be a limiting nutrient in the marine environment, although there are studies that point otherwise (*e.g.* Harrison *et al.*, 1990; Krom *et al.*, 1991). Phosphorus is considered to have structural and energy transfer functions. Is part of compounds such as adenosine triphosphate (ATP), guanine triphosphate (GTP), etc., nucleic acids, phospholipids and coenzymes. There are relatively few studies on phosphorus uptake kinetics in seaweeds. For *Porphyra*, few reports were found dealing with P uptake and tissue content. Wheeler and Björnsäter (1992) examined the variations in the tissue N and P content of several macroalgae, including *Porphyra* sp., and suggest that tropical macroalgae are consistently P limited. Hafting (1999) also established a relationship between the nutrient status of *P. yezoensis* in culture and the tissue N and P contents. If the blades were not N limited, a N:P supply ratio of 13-15 was enough to prevent P limitation. That author also concluded that *P. yezoensis* did not have the ability to store excess P over the range of nutrient provided. On the other hand, Chopin *et al.* (2004) reported the presence of cytoplasmatic polyphosphate granules in *Porphyra purpurea*. Flores-Moya *et al.* (1997) concluded that *Porphyra leucosticta* growing along the western Mediterranean sea was P limited during late winter – early spring season.

Since N is usually available in excess in integrated aquaculture conditions, it is important to understand if P might be limiting and if there is anything to gain with P enrichment. Chopin *et al.* (1999) estimated that N and P are released from fish aquaculture (salmon) in a proportion of 7:1. Chapter four of this thesis investigates the effects of P enrichment and lower N:P ratios on the growth and nutrient assimilation in *Porphyra dioica*.

Carbon dioxide

Seaweeds use inorganic carbon as virtually their sole carbon source (Lobban & Harrison, 1994). Seawater has inorganic carbon properties different from those in air or in fresh water. Compared to fresh water, its salinity and alkalinity are higher and its pH is higher and stable. Seaweeds in air can get inorganic carbon only as CO_2 that diffuses to them rapidly as they take it up. Seaweeds in seawater can also get CO_2 via the uptake of bicarbonate (HCO_3^-). However, HCO_3^- ions do not simply diffuse across the cell and chloroplast membranes. The concentration of total inorganic carbon in seawater, in equilibrium with air, is about 2.1 mmol l^{-1}, which is higher than in air and most freshwaters (Maberly, 1990). However, carbon dioxide concentration in air diffuses 10 000 times slower than in seawater (Maberly, 1990). In seawater of pH 8 and salinity 35‰, about 90% of the inorganic carbon is in the form of HCO_3^- (Lobban & Harrison, 1994). The relative proportions of the forms of inorganic carbon depend on pH, salinity and temperature. At atmospheric equilibrium the pH of seawater is close to 8.2 and the HCO_3^-/CO_2 ratio is about 150, but the concentration of dissolved CO_2 alters by approximately 10% for every pH unit near pH 8 (Menéndez *et al.*, 2001). In summer, higher temperature and higher salinity due to evaporation decreases CO_2 solubility.

Uptake of bicarbonate has been shown in a variety of seaweeds, both intertidal and subtidal, including fucoids, kelps, *Palmaria palmata*, *Chondrus crispus*, *Porphyra leucosticta* and an *Ulva* species (Beer & Eshel, 1983; Bidwell & McLachlan, 1985; Brechignac *et al.*, 1986; Surif & Raven, 1989; Mercado *et al.*, 1997). There are two ways in which seaweeds could actively use bicarbonate. In both ways, the enzyme

carbonic anhydrase (CA) would be used to convert HCO_3^- in CO_2. The two ways depend on the location where that enzyme acts, which is not known in most species. The first possibility is that seaweeds could actively uptake HCO_3^- through a specific transporter or by a general anion-exchange protein, being converted in CO_2 once inside the cell. The second possibility implies the use of extra-cellular CA, converting HCO_3^- in CO_2, which could then diffuse into the cells. Smith and Bidwell (1989) showed that *Chondrus crispus* uses HCO_3^- through the second mechanism. Zou *et al.* (2003) showed that the brown alga *Hizikia fusiforme* also lacks the capacity for active uptake of HCO_3^- but used it via extracellular CA. On the other hand, Choo *et al.* (2002) showed that *Cladophora glomerata* (Chlorophyta) has three possible HCO_3^- uptake mechanisms: 1) conversion of HCO_3^- into CO_2 via extracellular CA; 2) direct uptake via a DIDS-sensitive mechanism; and 3) by the involvement of a vanadate-sensitive P-type H^+-ATPase (proton pump). In *Porphyra*, activity of intra- and extracellular CA was detected in *P. leucosticta* and *P. linearis* (Mercado *et al.*, 1997; Israel *et al.*, 1999). Gao *et al.* (1992) found evidences for active HCO_3^- transport by *P. yezoensis* in that CO_2 uptake was extremely slow compared to the photosynthesis, and that external CA was never found in that species.

Maberly (1990) showed that the ability to use inorganic carbon was linked to the habitat in which the species grows. After testing 35 species of marine macroalgae, Maberly (1990) found that five of the six species which lack the ability to use HCO_3^-, grow subtidally and none in rockpools, where carbon depletion occurs. The ability of intertidal species to use HCO_3^- may appear surprising considering that they are exposed to air for considerable periods of time. This may, however, allow them to maximize their photosynthetic rates while underwater. It may also enhance the rates of photosynthesis in air before substantial desiccation occurs. Maberly (1990) suggested

that utilization of HCO_3^- by these intertidal species causes the pH in the water film to rise, thus promoting the diffusion of CO_2 from the air into the water film. Most *Porphyra* species, including those shown to use HCO_3^-, are intertidal, and therefore they seem to fit the hypothesis proposed by Maberly (1990).

OBJECTIVES

The objectives of this work were to increase the knowledge of the biology of *Porphyra dioica* Brodie *et* Irvine, one of the most common *Porphyra* species in the North of Portugal and possibly one of the most common species in the eastern North Atlantic Ocean (Holmes and Brodie, 2004). The initial objective of this thesis was to characterize the life history of this species, in the field and in the laboratory. Following the optimization of the control of the life cycle of *Porphyra dioica,* the second major objective was to determine the optimal conditions for the growth and productivity of the gametophytic phase and assess its potential for use in the bioremediation of nutrients in aquaculture.

The second chapter presents a study on the life history of *Porphyra dioica* from the Portuguese coast. Until my study, there was no published information available on the life history of this species. My study attempted to reveal the critical environmental factors that control the development of the gametophytic and sporophytic phases. All the blades used in the experiments were originated from a clonal conchocelis culture. This culture derived from a single zygotospore, isolated from a blade collected in Praia da Luz, Porto, Portugal. The aim was to eliminate or greatly reduce the influence of the

genetic variability on the responses to the culture conditions. The only possible genetic variation was eventually caused by meiosis.

The third and fourth chapters deal with the influence of the culture conditions on the growth of the blade phase of *Porphyra dioica*. In light of the potential application of species of this genus for integrated aquaculture and bioremediation, special attention was given to factors that maximized the production of the gametophytes. The third chapter attempts to analyze the influence of stocking density, temperature and photoperiod on the growth, productivity and nutrient uptake of *P. dioca*. The fourth chapter deals with N and P uptake. The first part of this chapter tested the influence of N concentrations and N sources on the growth of *Porphyra dioica* and on its N removal capacity. The second part of that paper deals with the diurnal uptake of two N sources (NO_3^- and NH_4^+). Finally, the ability of *P. dioica* to uptake phosphorus under different concentrations and N:P ratios was assessed also in chapter four. The results of the two last chapters give a perspective of the ability of *Porphyra dioica* to cope with high and fluctuating N and P concentrations. This thesis provides the basic knowledge needed to develop a pilot-scale cultivation of *P. dioica* in an integrated aquaculture environment.

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CHAPTER 2

Field and culture studies of the life history of *Porphyra dioica* (Bangiales, Rhodophyta) from Portugal

(Accepted for publication in Phycologia)

Field and culture studies of the life history of *Porphyra dioica* (Bangiales, Rhodophyta) from Portugal

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ABSTRACT

Aspects of the life history of *Porphyra dioica* Brodie *et* Irvine collected in Porto, Portugal, were investigated under laboratory and field conditions. This species is one of the most common *Porphyra* species on the North Coast of Portugal and can be found throughout the year. Field studies showed higher percentage cover, from 23 to 66%, during February through May (in 2001 and 2002), when compared to other times of the year. The effects of temperature, photon flux density (PFD) and photoperiod on growth and reproduction were tested. Zygotospores germinated fastest at 15ºC and a PFD of 25 µmol photons m^{-2} s^{-1}. Growth rate of the conchocelis was more affected by temperature than by photoperiod. In the three photoperiods tested, growth rate was maximal at 15ºC, under 25 to 75 µmol photons m^{-2} s^{-1}. Conchosporangia formation was greatest at 15ºC, 25 to 75 µmol photons m^{-2} s^{-1} light and short-day, 8:$\overline{16}$h L:$\overline{D}$ cycle conditions. In all conditions tested, conchosporangia formation was almost non-existent in free floating conditions. Optimal conditions for conchosporangia maturation (15ºC, 8: $\overline{16}$h L:$\overline{D}$ cycle and 5 to 25 µmol photons m^{-2} s^{-1}) also promoted spore release after 18 weeks. Aeration appeared to be crucial for normal blade development. No archeospores were observed. Preliminary findings of the conditions for growth of the gametophyte stage are also discussed.

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INTRODUCTION

Porphyra is a well-known seaweed genus due to its value as an aquaculture crop (Hanisak 1998). According to data from the Food and Agriculture Organization of the United Nations, production of *Porphyra* in 2001 was valued at 1.2 billion USD (FAO, 2003). Demand for *Porphyra* products, either as food or as commercial source of the red pigment r-phycoerythrin, appears to be growing (Levine 1998; Yarish *et al.* 1998), and a program is currently underway in New England (USA) to promote the domestication of indigenous *Porphyra* species for commercial cultivation (Yarish *et al.* 1998, 1999).

Since Drew's classical work (1954), many studies have dealt with the life histories of different species of *Porphyra* (Bird *et al.* 1972; Lewmanomont & Chittpoolkusol 1993; Notoya & Magaura 1998; Notoya & Miyashita 1999, among others). Different species have different requirements to complete their life cycles and to optimize growth and reproduction. The main triggers for life cycle events are temperature, photoperiod and photon flux density (PFD) (Krishnamurthy 1969; Kapraun & Lemus 1987, Waaland *et al.* 1987; Lewmanomont & Chittpoolkusol 1993; Nam-Gil 1999).

With our current understanding of this genus, we believe that there are at least five *Porphyra* species growing along the Portuguese coastline (Ardré 1970; South & Titley 1986; Brodie & Irvine 1997). *Porphyra dioica* Brodie *et* Irvine is one of the most common, and, because it can be found throughout the year, it may be a good candidate for aquaculture development. *Porphyra dioica* was described by Brodie & Irvine (1997) as differing from *Porphyra purpurea* (Roth) C. Agardh in being dioecious and forming packets of 8 zygotospores and 64 spermatangia whereas *P. purpurea* is monoecious (male and female regions separated by a distinct line) and typically has packets of 16

zygotospores and 64 to 128 spermatangia. Another distinctive characteristic is the distribution of the reproductive cells, along the margins in *P. dioica* and scattered in *P. purpurea*.

This paper describes some aspects of the population dynamics of the gametophyte and the effects of environmental conditions on zygotospore (*sensu* Nelson *et al.* 1999) germination and on growth, phenology and reproduction of the conchocelis phase of *Porphyra dioica*. The objective of this study was to provide basic knowledge of the biology of a Portuguese *Porphyra* species that may be useful for future aquaculture development.

MATERIAL AND METHODS

Field study

A population of *Porphyra dioica* was monitored during two years, in an area 20 km north of Porto, Portugal (41º19'37"N, 8º45'40"W). The percent cover and the presence of reproductive individuals were assessed monthly, between February 2000 and May 2002, in 13 quadrats of 0.25 m^2 each, fixed to the rocky substrate. Biomass was estimated based on the weight of the material collected in 10 quadrats of 0.04 m^2 each. The site was selected based on previous observations that a persistent bed of *Porphyra* existed in the area.

Zygotospore isolation

Female specimens of *Porphyra dioica* were collected from the coast at Porto (41º10' N, 8º 59'W) in September 2000. The surface of the blades was cleaned gently with a cotton ball using sterilized seawater. Small areas of reproductive tissue were excised and placed in Petri dishes with Von Stosch´s modified (Ott, 1965) enriched medium (VSE) plus 4 mg l^{-1} germanium dioxide (GeO_2) (within the range suggested by Lewin, 1966), and left overnight at 15ºC, 25 μmol photons m^{-2} s^{-1} and 12:$\overline{12}$ h L:$\overline{D}$ cycle. Light sources used in all experiments were cool-white fluorescent tubes. Liberated spores were transferred to new Petri dishes (100 mm diameter) containing VSE.

Germination experiments

Zygotospores were collected with a modified Pasteur pipette (the tip was drawn thinner in a Bunsen burner flame) and distributed into Petri dishes (60 mm diameter) at 25 and 75 μmol photons m^{-2} s^{-1} and at 5, 10, 15 and 20ºC, all at 12:$\overline{12}$ h L:$\overline{D}$ cycle. Three replicate plates per condition were observed periodically (daily until day 12 and then every 2-3 days), and the number of zygotospores germinating within the first 50 random spore observations was recorded. The percentage of germinating zygotospores through time was calculated and analyzed statistically with ANOVA (Rohlf and Sokal, 1981). A post-hoc analysis based on the ANOVA results was done with Student-Newman-Keuls (SNK) test to assess differences between temperatures (Rohlf and Sokal, 1981). An α equal to 0.05 was used for all the tests.

Conchocelis growth experiments

Conchocelis colonies derived from a single strain (PD2-1, Marine Biotechnology Laboratory, UCONN) were placed in 0.5 l flasks with VSE and gently aerated, under 15ºC, 30 ± 5 μmol photons m^{-2} s^{-1} and 12:$\overline{12}$ h L:$\overline{D}$ cycle, for 4 to 5 weeks to increase biomass. This strain originated from a single zygotospore from material collected in Porto, as described in the zygotospore isolation procedure. For these experiments, conchocelis were ground, using a common kitchen grinder, and passed through screen filters. The resulting filaments, between 50-70 μm in length, were suspended in seawater. One ml aliquots of this mixture were inoculated into Corning cell wells (6-well with lid) containing 10 ml of VSE and placed under different combinations of light (25 or 75 μmol photons m^{-2} s^{-1}), temperature (5, 10, 15 or 20ºC) and photoperiod (16:$\overline{8}$ h, 12:$\overline{12}$ h or 8:$\overline{16}$ h L:$\overline{D}$ cycle). The medium was changed weekly.

The growth of conchocelis under the experimental conditions was recorded weekly, as an increase in the area (α). Area was estimated using the formula:

$$\alpha = \frac{\pi}{n} \times \left(\sum_{i=1}^{n} \left(\frac{D_i}{2} \right)^2 \right)$$

This formula is based on the area of a circle, where the ray (D_i/2) is obtained from the mean of two perpendicular diameter measurements for each conchocelis tuft (D_i).

The area values were used to calculate specific growth rates (μ), as the mean percent increase per day, using the formula:

$$\mu = (\mathrm{Ln}\ (\alpha_2 / \alpha_1)) \times 100 / t$$

where α_2 and α_1 are the conchocelis area at the end and at the beginning of the experiment, and *t* is the number of days. This formula (DeBoer *et al.*, 1978) assumes that growth is exponential and was also used by Chopin *et al.* (1999) and by Stekoll *et al.* (1999) for conchocelis growth rate measurement.

The growth rates were analyzed using a three-way ANOVA to assess differences between temperature, photoperiod and photon flux density, and possible interactions. For this analysis, the growth rate value from week 2 to 4 of each replicate was used, since the first week was considered an adaptation period. The growth rates for 5ºC were not used because growth under short-day conditions was not tested at this temperature, and this would cause an asymmetry in the analysis. A post-hoc analysis was performed using SNK test for differences between temperature and between photoperiod treatments (Rohlf & Sokal, 1981). An α equal to 0.05 was used for all the tests.

Conchosporangia formation and conchospore release

The filaments grown under the conditions described above were also monitored for conchosporangia formation and conchospore release. The number of conchocelis with conchosporangia, within the first 30 randomly observed conchocelis in each well, was recorded weekly. The percentage of reproductive conchocelis was calculated. Simultaneously, vegetative conchocelis tufts from several conditions were transferred to different environmental conditions. Several combinations of changes in temperature, photoperiod and PFD were tested for conchosporangia formation and maturation. For these purposes, a PFD of 5 μmol photons m^{-2} s^{-1} was also tested. These conchocelis were also followed weekly for conchosporangia formation. Simultaneously, some

gently aerated cultures, maintained as a source of inoculum for other experiments, were also followed for conchosporangia formation.

The ability of the conchospores to attach to nets typically used in *Porphyra* cultivation was also assessed. Pieces of synthetic string were added to the cultures with mature conchosporangia, and the attachment of the conchospores was confirmed by observation using a dissecting microscope.

RESULTS

Field observations

Porphyra dioica can be found on the rocky shores of the north coast of Portugal throughout the year. It is often attached to a rocky substrate in the eulittoral zone above the area with *Fucus* spp. and below that of *Porphyra umbilicalis* (Linnaeus) Kützing, but may be found dispersed among these other algae. During the period of study, *P. dioica* revealed an amazing capacity to tolerate periods of sand covering lasting several days. The severity of these episodes sometimes prevented the assessment of percent cover (Fig. 1), when the quadrats were completely buried. That was the case in March and May 2000 (data for May 2000 is based only in one quadrate found). Nonetheless, subsequently the algae reappeared in high densities.

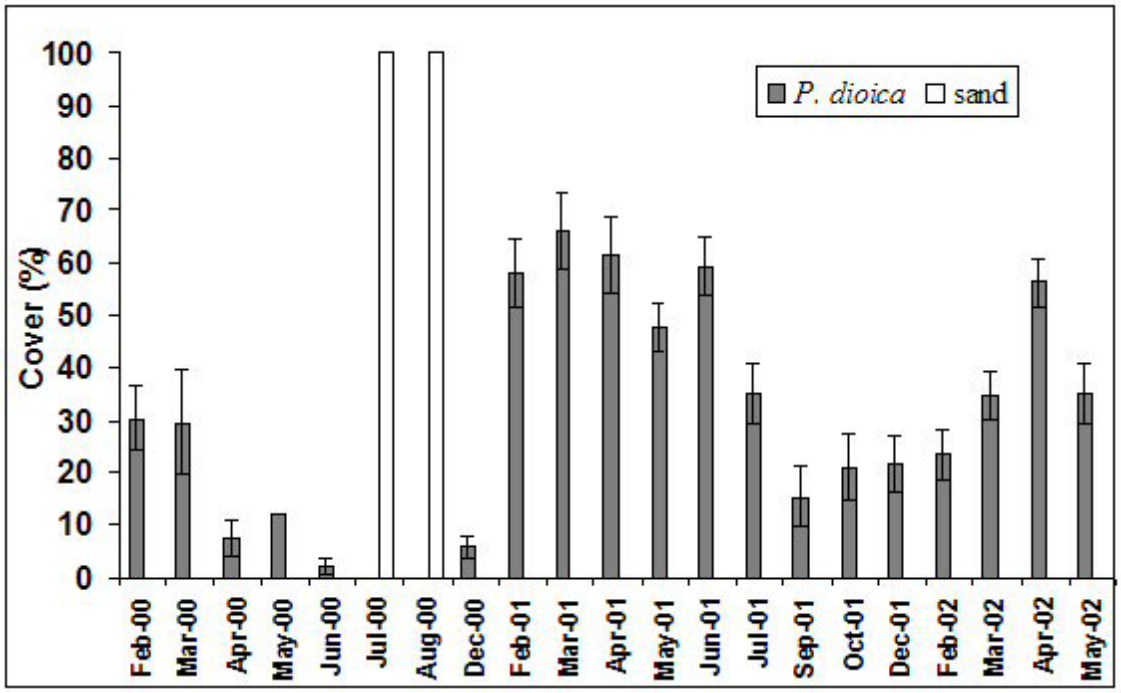


Figure 1 – Percent cover of *Porphyra dioica* on Mindelo beach, 20 km north of Porto. Bars represent the mean (± SD) of 9 to 13 0.25m^2 quadrats, affixed to the rock.

From February 2001 until May 2002 the percent cover of *Porphyra dioica* in Mindelo (20 km north of Porto) ranged from 15 to 66%, corresponding to 80 and 714 g fw m^{-2}, being higher during spring months (Fig. 1). Male and female individuals were found in the population all year.

The morphological characters of the specimens from which strains were isolated for life history studies are presented in Table 1 and Figs 2-9. The specimens had morphological and cytological characters that, in general, matched the description of this species (Brodie and Irvine, 1997). However, the diameter of vegetative cells of the Portuguese plants ranged from 9.9 to 21 µm compared to the 10 to 16 µm recorded by those authors. The zygotosporangia packets were larger in the specimens used in this work, 40-48 µm x 24-36 µm in surface view, versus the 19-22 µm x 17-20 µm of

Brodie and Irvine (1997). Species identification was confirmed by Chris Neefus using restriction fragment length polymorphism (RFLP) assay comparison, as described by Teasdale *et al.* (2002).

Table 1 – Morphological characters of *Porphyra dioica* specimens collected in Porto, Portugal.

	Vegetative thallus (μm)	Female thallus (μm)	Male thallus (μm)
Thallus thickness	1 cell layer: 52-65	65 to 76	70-74
Cell size (surface view)	10-21[1]	12-18 x 18-24[2]	4-7[3]
Cell height (cross section)	38-39	10-24	± 7.5
Cell length (cross section)	12-15	8-22	7-9
Arrangement		2x2x2	2x4x8
Packet size (surface view)		24-36 x 40-48[4]	13-17 x 20-25[5]

[1] Minimum and maximum cell length observed in cells in the centre of the thallus, away from the margins with reproductive or growing areas. Cells have irregular shapes.
[2] Minimum and maximum length and height of the cells in surface view. Cells are square to rectangular.
[3] Minimum and maximum diameters. Cells are round.
[4] Minimum and maximum packet length and height in surface view. Packets are slightly rectangular.
[5] Minimum am maximum packet length and height in surface view. Packets are rectangular.

Figures 2-9: Morphology of *Porphyra dioica* from Porto, Portugal. Scale bars = 20 µm (Figs 4-6, 8, 9) or 40 µm (Figs 3, 7).

Fig. 2. Mature female specimen.
Fig. 3. Cross section of vegetative area showing one cell layer.
Fig. 4. Surface view of vegetative cells.
Fig. 5. Surface view of spermatangia.
Fig. 6. Cross section of spermatangia.
Fig. 7. Cross section of zygotosporangia.
Fig. 8. Surface view of zygotosporangia.
Fig. 9. Zygotospore.

Zygotospore germination

The germination rates of the zygotospores were higher at 15 and 20ºC than at other temperatures regardless of the PFD (25 or 75 µmol photons m^{-2} s^{-1}; Fig. 10). According to the ANOVA using germination data from day 20, there was no significant difference between 25 and 75 µmol photons m^{-2} s^{-1} ($P > 0.05$) nor was there any interaction between light and temperature. On the other hand, there was a significant difference among temperatures ($P < 0.01$). The SNK test showed that there were differences in germination between 5-10ºC and 15-20ºC but not within these temperature pairs.

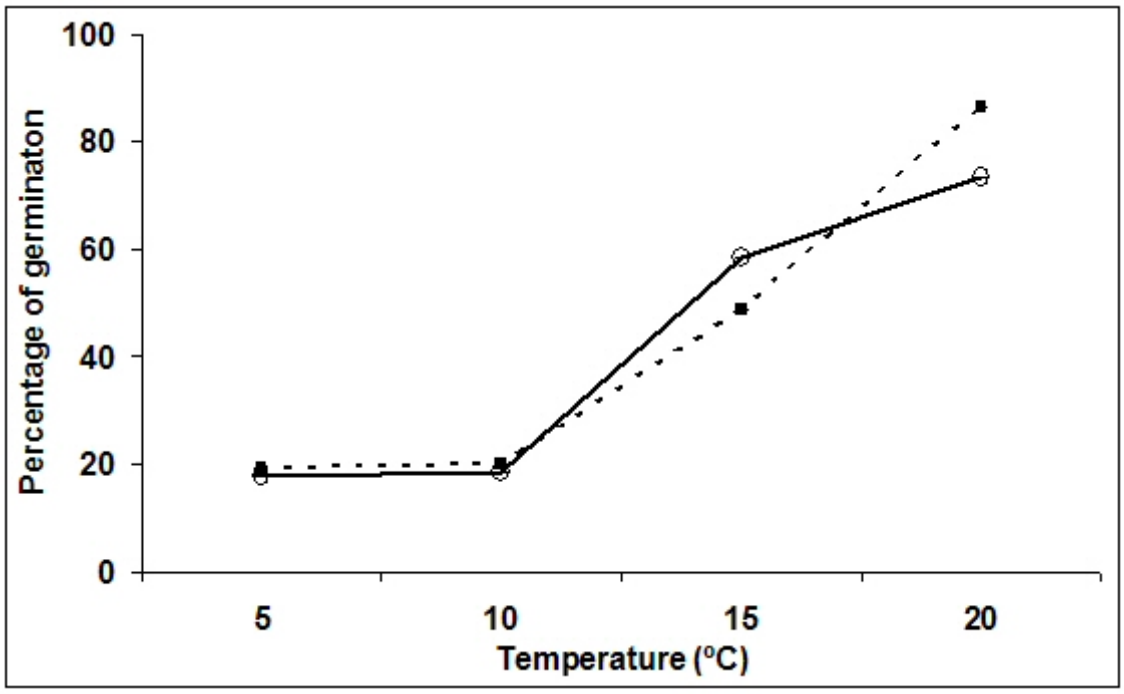


Figure 10 – *Porphyra dioica*. Percent germination of zygotospores after 20 days in culture under different temperatures (5, 10, 15, 20ºC) and PFDs (■, 25; ○, 75 µmol photons m^{-2} s^{-1}). Germination was calculated using the first 50 random observations of spores. Values represent means of 3 replicates with corresponding standard error (error bars to small to be seen in the graphic).

The change in germination rates through time (Fig. 11) combines data from 15 and 20ºC and compares only the two PFDs (since there was no difference in the

germination rates between these two temperatures). At 25 μmol photons m^{-2} s^{-1} 40% germination was recorded on the third day. However, these high germination rates declined during the following days and, on the eighth day, were not significantly different ($P > 0.05$) from those at 75 μmol photons m^{-2} s^{-1}. At 75 μmol photons m^{-2} s^{-1} only after 11 to 15 days was germination higher than 10% (Fig. 11). At 20ºC and 25 μmol photons m^{-2} s^{-1} germination levels of 90% were achieved after 22 days, although this value is not significantly different from the 77% at 15ºC and the same PFD ($P > 0.05$). At higher PFD, levels of 90% germination were recorded only on day 30.

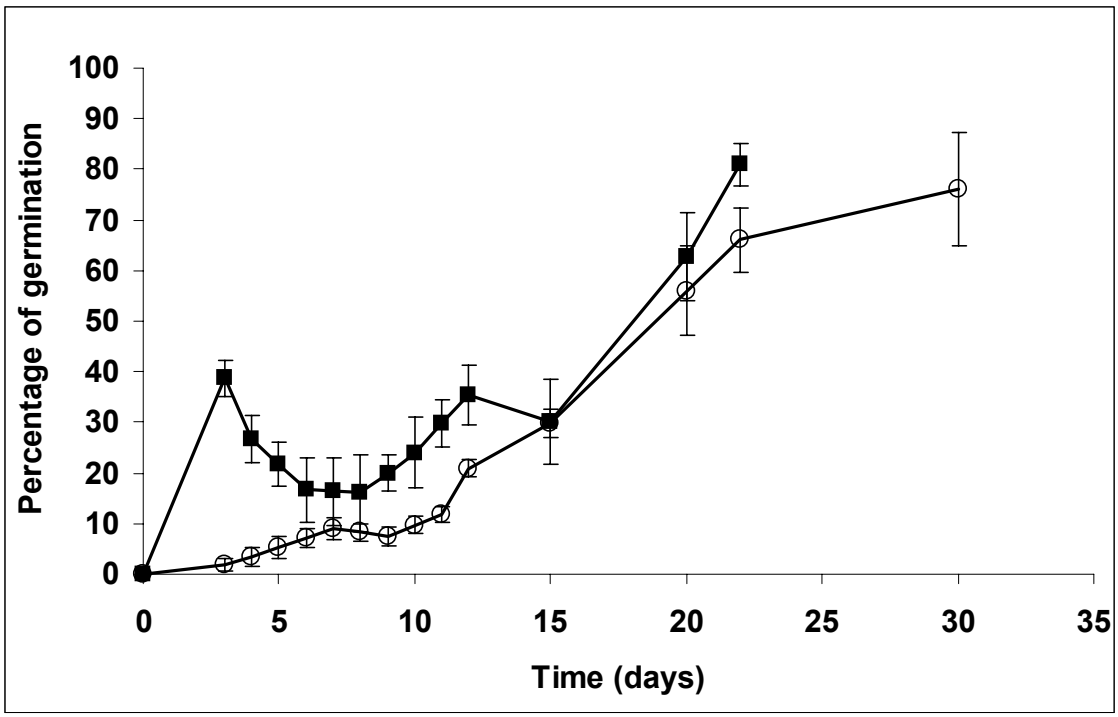


Figure 11 – *Porphyra dioica*. Change in mean percent germination of zygotospores obtained at 15 and 20ºC under neutral-day conditions as a function of PFD (■, 25; ○, 75 μmol photons m^{-2} s^{-1}). Germination calculated from the first 50 random observations, 6 replicates per condition, with corresponding standard error.

The influence of temperature on germination was also confirmed through transfering zygotospores to different temperatures. At 25μmol photons m^{-2} s^{-1},

zygotospores initially grown at 5 and 10ºC were transferred to 20ºC on day 22. This caused an increase in the germination rate up to 60 and 89%, respectively, on day 30. This indicates that temperatures as low as 5ºC, below the values recorded in Portuguese coastal waters, are not lethal for zygotospores at least for up to 22 days of exposure.

Conchocelis growth

Well-developed conchocelis tufts were regenerated, in all conditions, starting from mostly single unbranched filaments 50-70 μm in length. No protothalli were produced. No occurrence of neutral conchospores or conchocelis archeospores (*sensu* Nelson *et al.* 1999) was noted. The cells of the filament were usually 7.5 ± 2.4 μm in diameter and 76.6 ± 7.4 μm long, and no differences in these characters were noted under any of the growth conditions. On the other hand, the conchocelis produced many more ramifications when grown at 15 and 20ºC, regardless of the photoperiod and PFD. While the conchocelis at 5ºC did not grow much until the fourth week, those at 10ºC grew well but were less branched than the ones at 15 and 20ºC (data not shown). This difference gradually disappeared during the following 4-5 weeks. The conchocelis at 10ºC then acquired a morphology similar to the conchocelis at the higher temperatures.

The average growth rate of the conchocelis was higher at 15ºC in all photoperiods, independent of irradiance tested (Figs 12, 13). At 15 and 20ºC, the average area increase was always more than 20% day^{-1}, regardless of the photoperiod. During the same period, for 10 and 5ºC the maximum mean growth rates recorded were 18.1 and 6.7%, respectively.

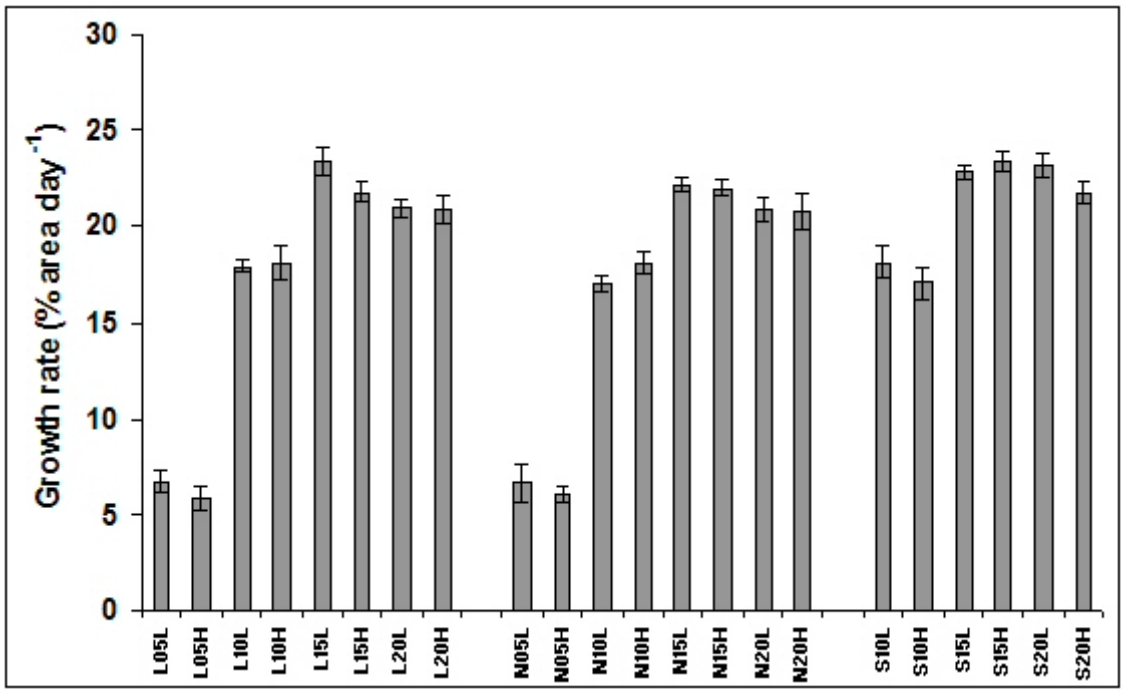


Figure 12 – *Porphyra dioica*. Growth rates of conchocelis under a combination of photoperiods, PFDs and temperatures. L, N or S stands for 16:$\overline{8}$, 12:$\overline{12}$ and 8:$\overline{16}$h L:$\overline{D}$ cycles, respectively; 05, 10, 15 or 20 stands for the temperature in ºC; L or H stands for Low-PFD (25 µmol photons m^{-2} s^{-1}) or High-PFD (75 µmol photons m^{-2} s^{-1}), respectively. Bars represent means, after 21 days in culture, of 6 replicates with corresponding standard error.

According to the three-way ANOVA, there were differences among temperatures ($P < 0.001$) and among photoperiods ($P < 0.05$) but not between the two PFDs tested ($P > 0.05$), and there were no interactions between any of the factors. The SNK test revealed that growth rates at 5ºC were clearly different from those at all other temperatures. In respect to photoperiod, the SNK test detected a difference between growth rates at 8:$\overline{16}$h L:$\overline{D}$ cycle in relation to those at 12:$\overline{12}$h L:$\overline{D}$ cycle, but not between 12:$\overline{12}$h L:$\overline{D}$ and 16:$\overline{8}$h L:$\overline{D}$ cycle, nor between 8:$\overline{16}$h L:$\overline{D}$ and 16:$\overline{8}$h L:$\overline{D}$ cycle.

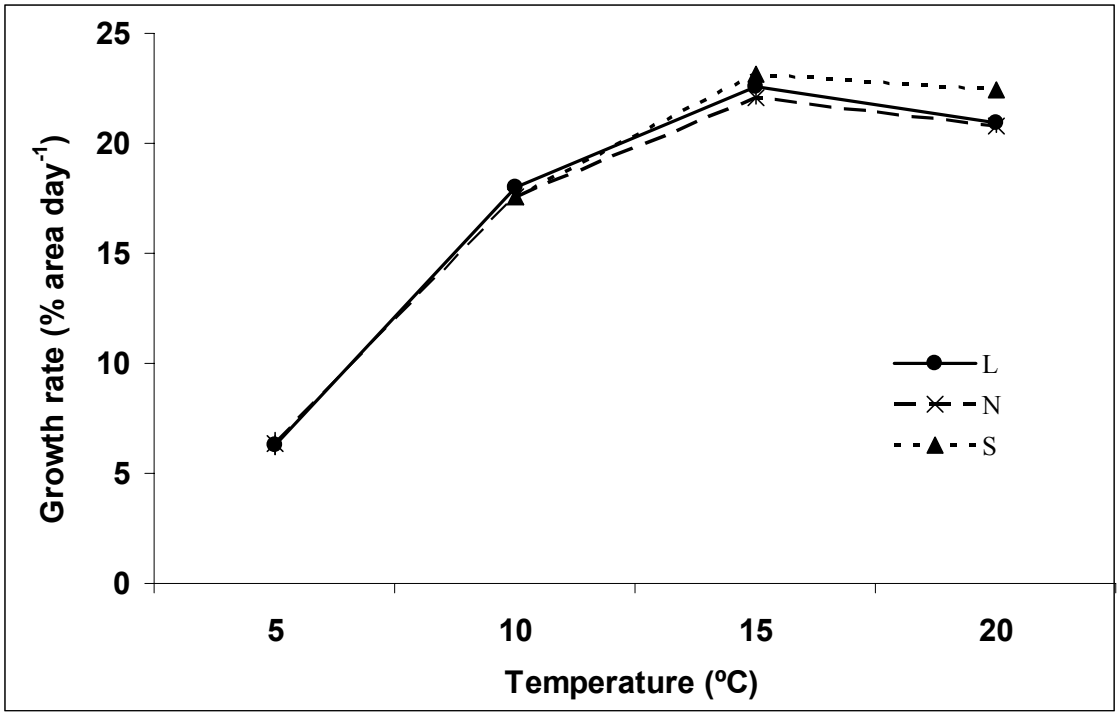
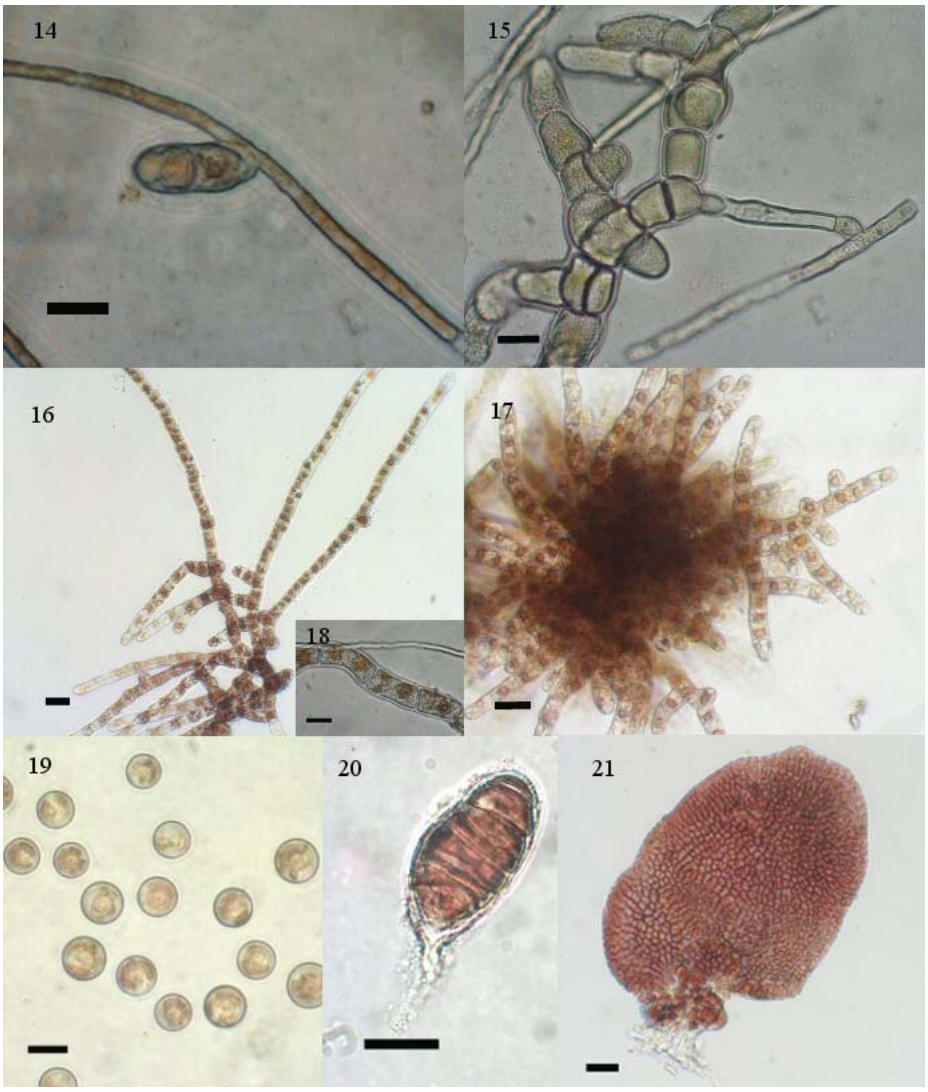


Figure 13 - *Porphyra dioica*. Average growth rates of conchocelis under a combination of photoperiods, PFDs and temperatures. L, N or S stands for 16:$\overline{8}$, 12:$\overline{12}$ and 8:$\overline{16}$ h L:$\overline{D}$ cycles, respectively; dots represent means after 21 days in culture of 12 replicates with the correspondent standard error (error bars to small to be noted in the graphic).

Conchosporangia formation and conchospores release

Conchosporangial branches can form either from the apical cell of a vegetative filament or laterally from cells in the middle of a vegetative filament (Figs 14, 15). The morphology of the conchosporangial tufts differed depending on the substrate. Those growing on shells were not as branched (Fig. 16) as those attached to the bottom of Petri dishes (Fig. 17). The conchosporangial branches in the *Porphyra dioica* conchocelis were 20 ± 1 μm in diameter. In lateral view, the cells were square to rectangular (up to 37.8 μm long) when immature (Fig. 18). Released conchospores were 20 ± 2.5 μm in diameter (Fig. 19) and germinated to form new thalli. The young blades were approximately 53 μm long after 3 days and more than 500 μm long after 3.5 weeks (Figs 20, 21).



Figures 14-21: Details of different stages in the life cycle of *Porphyra dioica* in culture. Scale bar = 20 µm (Figs 14, 15, 18-20), 40 µm (Figs 16, 17) or 50 µm (Fig. 21).

Fig. 14. Conchosporangial branch arising laterally from a vegetative filament, after 8 weeks at 15ºC and 25µmol photons m^{-2} s^{-1} under $8:\overline{16}$ h L: $\overline{D}$ cycle.

Fig. 15. Conchosporangial branch formed from the apical cell of a vegetative filament, same conditions as Fig. 14.

Fig. 16. Conchosporangial tuft formed by conchocelis growing on shell.

Fig. 17. Conchosporangial tuft formed by conchocelis growing on the surface of a Petri dish.

Fig. 18. Detail of conchosporangial cells.

Fig. 19. Released conchospores.

Fig. 20. Young thallus after 3 days at 15ºC and 25 µmol photons m^{-2} s^{-1} under $16:\overline{8}$ h L: $\overline{D}$ cycle.

Fig. 21. Blade after 3.5 weeks.

Conchosporangia were formed mainly in non-aerated cultures but also in aerated cultures with attached conchocelis. In aerated cultures with free floating conchocelis some larger cells were observed, but they never formed typical conchosporangia. In stationary cultures, the first conchosporangia were observed 6 weeks after germination of the zygotospores, at 15ºC, $8:\overline{16}$h L:$\overline{D}$ and $12:\overline{12}$h L:$\overline{D}$ cycle, but also at 20ºC, $12:\overline{12}$h L:$\overline{D}$ and $16:\overline{8}$h L:$\overline{D}$ cycle. However, after 15 weeks, it was clear that the higher frequencies of conchosporangia formation were achieved at 15ºC and $8:\overline{16}$h L:$\overline{D}$ cycle, without significant difference between the two PFDs (Fig. 22). Under these conditions, around 77 and 83% of the conchocelis formed conchosporangial branches at 25 and 75 µmol photons m^{-2} s^{-1}, respectively. In $12:\overline{12}$h L:$\overline{D}$ cycle, at 10ºC the frequencies were around 58% for both PFDs, whereas at 15ºC the frequencies were 62 and 22% for 25 and 75 µmol photons m^{-2} s^{-1}, respectively.

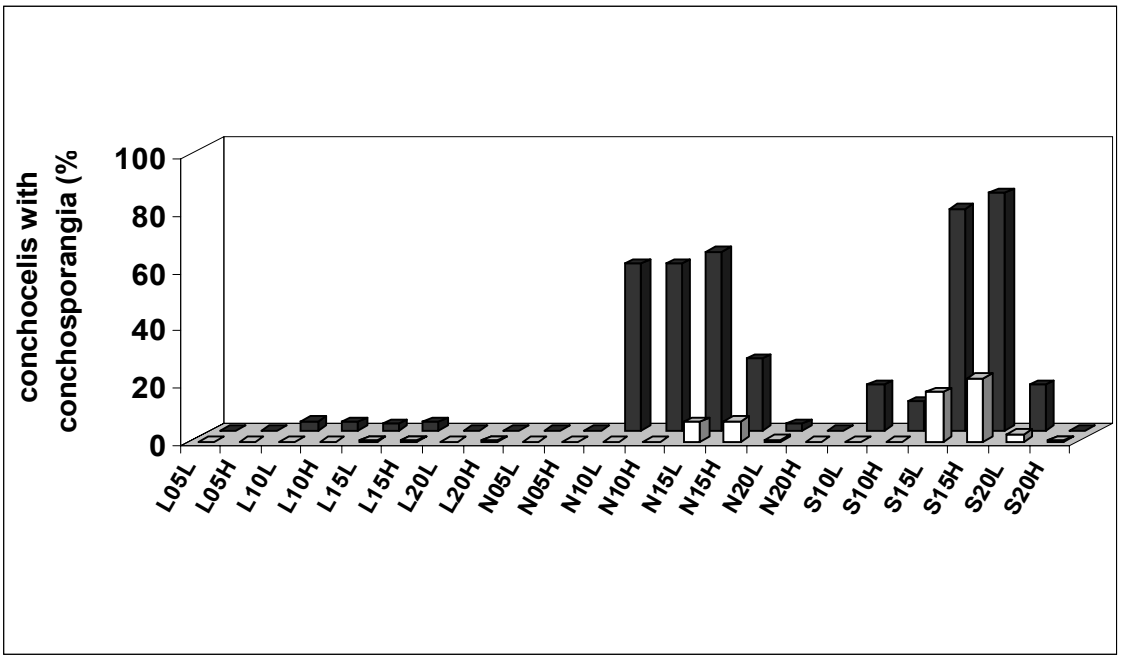


Figure 22 - *Porphyra dioica*. Percent conchocelis with conchosporangia after 7(white columns) and 15 weeks (dark columns) in culture, under a combination of photoperiods, PFDs and temperatures. L, N or S stands for $16:\overline{8}$, $12:\overline{12}$ and $8:\overline{16}$h L:$\overline{D}$ cycles, respectively; 05, 10, 15 or 20 stands for the temperature in ºC; L or H stands for Low-PFD (25 µmol photons m^{-2} s^{-1}) or High-PFD (75 µmol photons m^{-2} s^{-1}), respectively. Columns represent the average of 6 replicates in each condition, using the first 30 random observations in each replicate.

At 20 ºC and 8:$\overline{16}$h L:$\overline{D}$ cycle, the frequencies recorded were not reliable. In these conditions we experienced problems with contaminants that prevented normal development of the conchocelis. The 16% value for 20ºC and 8:$\overline{16}$h L:$\overline{D}$ cycle at 25 µmol photons m^{-2} s^{-1} is based only on one of the six replicates.

There is a clear difference in the frequency of conchosporangia formation when the conchocelis were transferred to an 8:$\overline{16}$h L:$\overline{D}$ cycle, in relation to those transferred to a 12:$\overline{12}$h and a 16:$\overline{8}$h L:$\overline{D}$ cycle (Table 2). In the transfers to or within an 8:$\overline{16}$h L:$\overline{D}$ cycle, only the ones moved from 15ºC to 10ºC did not produce any conchosporangia. At 20ºC, conchocelis transferred from a 12:$\overline{12}$ to an 8:$\overline{16}$h L:$\overline{D}$ cycle formed conchosporangia with a frequency of 36.6% after 12 weeks.

Table 2 - Frequency of conchocelis with conchosporangia 12 weeks after the transference of 4.5 week old cultures to different photoperiods, temperatures or both. Values are the mean of 2 replicates for each new condition and the frequency is based on the first 30 random observations. N, S and L, 12:$\overline{12}$, 8:$\overline{16}$ or 16:$\overline{8}$h L:$\overline{D}$ cycles, respectively. 10, 15 and 20, temperature in ºC. L and H, 25 and 75 µmol photons m^{-2} s^{-1}, respectively.

From N to (or within) Short-day			From N to (or within) Long-day			From L or S to (or within) Neutral-day		
Origin	New	%	Origin	New	%	Origin	New	%
N15L	S15L	11.6	N15L	L15L	0.0	S15L	N15L	0.0
N20H	S15H	35.5	L15H	L15L	0.0	L15H	N15L	0.0
S20H	S15H	20.0	N15L	L15H	0.0	S15L	N15H	6.6
S15L	S10L	0.0	N15H	L15H	0.0	S15H	N15H	1.6
N20H	S20H	36.6	N15H	L20H	0.0	L15H	N15H	6.6
			L15H	L20H	0.0	N20H	N15H	15.0
			N20H	L20H	1.6	N10H	N15H	5.0
						N15L	N10L	0.0
						N15H	N20H	0.0
	Average	20.7			0.2			3.8

A very low percentage (15% or less) of conchocelis formed conchosporangia when transferred to a 12:$\overline{12}$h L:$\overline{D}$ cycle. This frequency was even smaller when the conchocelis were transferred to a 16:$\overline{8}$h L:$\overline{D}$ cycle. It is interesting to note, however, that the only changes in cultures transferred to 15ºC and a 12:$\overline{12}$h L:$\overline{D}$ cycle were observed at 75 µmol photons m^{-2} s^{-1}. These cultures were originally at the same temperature but different photoperiods or under the same photoperiod but at different temperatures. The same transfers but to 25µmol photons m^{-2} s^{-1} did not produce any conchosporangia. These results are different from those observed in cultures not subjected to any change in conditions, where there was no difference between the two light intensities. In any case, in the cultures transferred to or within neutral-day, the highest frequency of conchocelis with conchosporangia was only 15%.

Conchospores of *Porphyra dioica* (Fig. 19) were first released, after 18.1 weeks, only at 15ºC and 8:$\overline{16}$h L:$\overline{D}$ cycle. These conchocelis were maintained at 5 µmol photons m^{-2} s^{-1} for 9 weeks, followed by an increase in light and the introduction of aeration. Although the conchosporangia were formed under 25 and 75 µmol photons m^{-2} s^{-1}, a period of 9 to 10 weeks under 5 µmol photons m^{-2} s^{-1} seems to promote faster maturation of the conchospores and release even without aeration. Conchosporangia transferred back to 25 µmol photons m^{-2} s^{-1}, after 9 weeks at 5 µmol photons m^{-2} s^{-1}, also released spores at the same time or slightly sooner if aerated.

When the conchosporangia were transferred from 15ºC, 8:$\overline{16}$h L:$\overline{D}$ cycle and 25 µmol photons m^{-2} s^{-1} to other conditions, only the ones at 10ºC, same photoperiod and light, released spores and generated blades. No released spores were observed in any of the other conditions tested (Table 3).

Table 3 – List of other conditions tested, not suitable for conchospore release.

Temperature (ºC)	Photoperiod (hours, L:$\overline{D}$)	PFD (µmol photons m^{-2} s^{-1})	Aeration	Time in these conditions (weeks)
10	8:$\overline{16}$	25	No	6.6
10	8:$\overline{16}$	5	No	4.6
10	12:$\overline{12}$	25	No	6.6
10	12:$\overline{12}$	25	Yes	6.6
10	12:$\overline{12}$	5	Yes	4.6
10	12:$\overline{12}$	10	No	4.6
15	8:$\overline{16}$	25	No	6.6
15	12:$\overline{12}$	25	No	6.6
15	12:$\overline{12}$	25	Yes	6.6
15	12:$\overline{12}$	5	No	4.6
15	12:$\overline{12}$	5	Yes	4.6
20	8:$\overline{16}$	25	No	6.6
20	8:$\overline{16}$	10	No	4.6
20	8:$\overline{16}$	25	Yes	6.6
20	12:$\overline{12}$	25	No	6.6
20	12:$\overline{12}$	25	Yes	4.6

All the conchosporangia transferred to these conditions were 10 weeks old and formed at 15ºC, 8:$\overline{16}$ h L:$\overline{D}$ and 25 µmol photons m^{-2} s^{-1}.

Conchosporangia maintained at 15ºC, 8:$\overline{16}$ h L:$\overline{D}$ cycle, 25 µmol photons m^{-2} s^{-1} and aerated, released spores that attached to the bottom and walls of the flask and formed young blades (Figs 20, 21), first visible 21.6 weeks after the beginning of the experiment and 4.6 weeks after aeration was started. Young blades were also visible in cultures transferred from 15 to 10ºC, still in 8:$\overline{16}$ h L:$\overline{D}$ cycle and 25 µmol photons m^{-2} s^{-1}, with aeration, 6.6 weeks after the transfer and the start of aeration. Under these

conditions the exact timing of the spores release was not determined. The conchocelis were free-floating in 1 l flasks and released conchospores were never observed. We assume that the release occurred between the 18th and 20th weeks in culture and around 3 weeks after the beginning of aeration.

Another option for spore release is to use the conchosporangia produced in 15ºC and 12:$\overline{12}$ L:$\overline{D}$ cycle. These cultures had mature conchosporangia after 19 weeks and released conchospores but only 1-2 weeks after transfer to 15ºC or 10ºC and 8:$\overline{16}$ L:$\overline{D}$ cycle. Optimal conditions for the growth and reproduction of the conchocelis stage are summarized on Table 4.

Table 4- Summary of the best conditions for growth and reproduction of the conchocelis of *Porphyra dioica*.

	Temperature (ºC)	Photoperiod (hours, L:$\overline{D}$)	PFD (µmol photons m^{-2} s^{-1})	Time (weeks)
Growth of conchocelis	15-20	8:$\overline{16}$ 12:$\overline{12}$ 16:$\overline{8}$	25-75	4-6
Conchosporangia formation [1]	more at 15 also at 10	more at 8:$\overline{16}$ also at 12:$\overline{12}$	25-75	6-7
Conchosporangia maturation	15	8:$\overline{16}$ 12:$\overline{12}$	5[2]-25	± 10
Conchospore release	10[3] and 15	8:$\overline{16}$	25-75	1-2 weeks when transferred from 12:$\overline{12}$ to 8:$\overline{16}$

[1] Formation occurred only in non-aerated cultures or in cultures aerated but with the conchocelis fixed to the glass or to shell.
[2] Maturation seems to be promoted under 5 µmol photons m^{-2} s^{-1}
[3] Release at 10ºC and 8:$\overline{16}$ h L:$\overline{D}$ cycle occurred in cultures transferred from 15ºC, both 8:$\overline{16}$ and 12:$\overline{12}$ h, L:$\overline{D}$ cycles and aerated.

Young blades were not obtained from the spores released at 15ºC, 8:$\overline{16}$ L:$\overline{D}$ cycle, 5 and 25 µmol photons m^{-2} s^{-1} and non-aerated. Only two spores were observed germinating under each of these conditions, but none of them developed into a macroscopic blade. Conchospores transferred from 25 to 75µmol photons m^{-2} s^{-1}, within that same photoperiod, as well as conchospores transferred from 8:$\overline{16}$ to 12:$\overline{12}$ L:$\overline{D}$ cycle, 25 and 75 µmol photons m^{-2} s^{-1}, always at 15ºC and kept without aeration, did not germinate.

Conchospores attached to synthetic string commonly used for nori cultivation, as well as to PVC tubing and glass. Germination and subsequent formation of gametophytes occurred on all three substrates. No archeospores or any other kind of asexual spore (*sensu* Nelson *et al.* 1999) were produced by the young blades in culture.

DISCUSSION

The germination rate of the zygotospores of *Porphyra dioica*, under neutral day, is higher at 15ºC and 20ºC. Despite the clear advantage of the use of temperatures between 15ºC and 20ºC, the growth rate of the conchocelis at 10ºC cannot be neglected. Conchocelis cultivated at 10ºC grow and acquire normal morphology, although this happens slowly and the conchocelis initially have fewer ramifications than the ones grown at higher temperatures. The results also show that 5ºC delays, but does not stop, growth of the conchocelis after a period of acclimation. The conchocelis can even continue to grow during the first week after being transferred. This indicates an

acclimation capacity to temperatures below those that are usual in Portuguese coastal waters.

Germination rates of zygotospores were independent of the PFD, after 20 days, within the range tested. Although the first impression is that the lower light promotes faster germination, the high germination values after only three days rapidly decline. After one week the difference between the two light levels is not significant. This variation is probably explained by death of some of the first spores to germinate. Despite this, 25µmol photons m^{-2} s^{-1} should be preferred over the higher irradiance for zygotospores germination and isolation of conchocelis. Besides the energy saving, which may not be critical at the laboratory level but is important at larger scales, conchocelis at 25µmol photons m^{-2} s^{-1} were generally more pigmented.

This result shows that the conchocelis of *Porphyra dioica* does not require high PFD, as was observed in other *Porphyra* species. Waaland *et al.* (1990) found that the conchocelis of *P. abbottae* V. Krishnamurthy, *P. nereocystis* C.L. Anderson, *P. fallax* S.C. Lindstrom & K.M. Cole *(*as *P. perforata* J. Agardh; Lindstrom & Cole 1990*)*, *P. pseudolanceolata* V. Krishnamurthy and *P. torta* V. Krishnamurthy, from Washington State, USA, all grew well from 5 µmol photons m^{-2} s^{-1} to 100 µmol photons m^{-2} s^{-1}, with growth light saturated at 5 µmol photons m^{-2} s^{-1}in some cases. In species like *P. amplissima* (Kjellman) Setchell & Hus [as *P. miniata* (C. Agardh) C. Agardh; Lindstrom & Coll 1993], the conchocelis also displays a wide tolerance to light intensities, growing from 200 to 5000 lux, equivalent to 3.6—90 µmol photons m^{-2} s^{-1} (Chen *et al.* 1970). An interesting case is that of the conchocelis of *P. abbottae*. Stekoll *et al.* (1999), using a culture from Alaska, reported growth inhibition in light over 40 µmol photons m^{-2} s^{-1} at 15ºC or higher temperature, whereas the conchocelis from the same species collected in Washington was found by Waaland *et al*. (1990) to show good

growth in the range 10-15ºC and 5-100 µmol photons m^{-2} s^{-1}. The only difference between the two cultures was their origin. This example emphasizes the need for independent investigations of species already studied but collected from distant locations and probably adapted to local conditions.

There are not many studies that compare conchocelis growth under different photoperiods. The gametophytes of most *Porphyra* species are seasonal, and the conchocelis phase typically occurs during the opposite season or during the season that precedes the gametophyte season. Therefore, previous research usually has focused more on temperature and other factors, using photoperiod only to trigger spore formation and release (*e.g.* Chiang & Wang 1980; Waaland *et al.* 1990; Lewmanomont & Chittpoolkusol 1993; Nam-Gil 1999; Stekoll *et al.* 1999). Due to the year-round presence of the gametophytes of *P. dioica*, we were interested in finding what happens to the sporophyte under different photoperiods. The conchocelis of *P. dioica* showed growth rates higher than 20% d^{-1} in all photoperiods between 15ºC and 20ºC and grew also between 5ºC and 10ºC. This result suggests that the conchocelis of *P. dioica* can be present throughout the year on the north Portuguese coast. Notoya and Miyashita (1999) found a similar result for *P. moriensis* H. Ohmi, growing equally well at $10{:}\overline{14}$ and $14{:}\overline{10}$ h L:$\overline{D}$ cycles, 15-25ºC and surviving from 5ºC to 25ºC.

In regard to conchosporangia formation and release, it is possible to find species with very specific requirements, others that form conchosporangia in a wide range of conditions and others that need a sequence of changes in conditions. The main factor is photoperiod, although a more or less specific combination of temperature and irradiance is required, depending on the species. Species like *Porphyra abbottae*, *P. fallax* (as *P. perforata*), *P. pseudolanceolata* and *P. nereocystis* (Waaland *et al.* 1990), all respond to photoperiod but have different temperature and light requirements. *Porphyra abbottae*

produces conchosporangia from 5-300 µmol photons m^{-2} s^{-1} but needs temperatures between 12-15ºC. *Porphyra nereocystis*, on the other hand, is less temperature-specific, 10-15ºC, but requires exactly 10 µmol photons m^{-2} s^{-1}. Other species, like *P. torta* (Waaland *et al.* 1990), *P. columbina* Montagne (Avila *et al.* 1986) and *P. leucosticta* Thuret (Sidirelli-Wolff 1992) respond to two different photoperiods. An interesting species, which shows a total absence of response to photoperiod for conchosporangia formation, is *P. angusta* Okamura & Ueda (Chiang & Wang 1980). This species has a frequency of conchosporangia formation of 100%, or very close to it, as long as cultures are at 27-29ºC, but that report does not give any information on the requirements for conchospore release.

Porphyra dioica is able to form conchosporangia in all photoperiods tested, from 10 to 20ºC and from 25 to 75 µmol photons m^{-2} s^{-1}, always without aeration. No conchosporangia were observed in free floating cultures. This result is hard to explain since, obviously, there are no still waters in the natural habitat. On the other hand, conchocelis aerated while attached to the flask or to pieces of shell, thus not free floating, did produce conchosporangia. There are other reports of species that rarely, if ever, form reproductive structures if the plants are not attached (Lobban & Harrison 1994). Norton & Mathieson (1983) reject the lack of a fixed orientation as the cause for this infertility. Instead they suggest, between other possibilities, that unattached seaweeds become locked into a juvenile stage, in which they are unresponsive to the environmental factors that typically would trigger reproduction.

The frequencies of conchosporangia formation obtained under neutral-day, 10 and 15ºC should not be neglected. Although conchosporangia formation is not very condition-specific, maturation and release occur preferentially under a combination of short-days and 15ºC. This is also the condition that produces more conchosporangia,

thus making photoperiod and temperature important factors. The observations during this work suggest that the kind of culture (free floating or fixed) and substrate influence the frequency of conchosporangia formation and also the maturation and release. There is almost complete absence of conchosporangia in free floating cultures. Moreover, conchocelis grown on shells seem to produce more conchosporangia than those grown on glass. This is based on simple observation, and no quantitive data are available at present. Further studies are needed, specifically designed to understand the possible interactions between the kind of substrate, water motion, and the frequency of conchosporangia formation.

The different branching patterns of the conchosporangial tufts are probably related to the direction from which the filaments receive light. Filaments growing attached to shells were clearly less branched than those attached to glass, as mentioned earlier. The filaments growing on glass receive light from all directions, except the bottom, whereas the ones on the shells seem to grow towards the light, received mainly from above. However, we have no explanation as to how light direction affects branching.

Besides temperature and photoperiod, light intensity plays an important role in maturation of the conchospores. Our results show that a period of 9 weeks at 5 µmol photons m^{-2} s^{-1} promotes maturation and release of conchospores. This happens without water movement, apart from that unavoidably caused by handling the cultures. Conchospores also matured and were released in the cultures at 25 µmol photons m^{-2} s^{-1} when aeration was provided.

Blades were only obtained at 10-15ºC with aeration. The temperature range for blade formation agrees with that found on the north coast of Portugal during the months of higher abundance of gametophytes in the field, *i.e.*, February to April. The first

attempts to grow blades showed that they grew under all three photoperiods, at least at 15ºC.

The wide tolerance of the zygotospores and conchocelis to the range of temperatures tested allows for the occurrence of conchocelis throughout the year, just as happens with the gametophyte. However, a continuous production of spores and constant renewal of the gametophyte population is unlikely to occur. Besides the fact that the presence of the conchocelis phase in nature has not been confirmed by us, conchospore release in the laboratory required a particular combination of temperature, photoperiod and PFD. These observations preclude the possibility of a constant renewal of gametophytes in nature. Our field work also shows that there is a clear increase in the population during winter and early spring months. With a constant renewal of the population, we would expect percent cover to be more stable throughout the year. On the other hand, the fact that we were able to grow blades under all three photoperiods supports the idea of a possible continual renewal of the population. The possibility of a constant renewal of the population, maybe with different intensities through the year, cannot be excluded. In fact, Holmes & Brodie (2004) also report the occurrence of *Porphyra dioica* throughout the year at Sidmouth Bay (Devon, UK), with young blades preent during all year, suggesting a constant renewal of the population. In that study the highest proportion on individuals that were <1.00 g wet mass were recorded in February. This aggress with the period of increase in the percent cover (late winter to spring months) recorded in our study, preceded by the period in which natural conditions macth those that in the lab resulted in conchospore release.

One explanation for the presence of gametophytes in nature during all year could be the formation of archeospores or other kind of asexual spores, although such spores were never observed in the laboratory. Also, there is not, to our knowledge, information

on how long gametophytes can last in nature. From our experience, blades produced in the lab grow for several weeks (or up to two months), and then reproduction is followed by disintegration.

In conclusion, our results show that the main factor controlling the growth of conchocelis is temperature, assuming that nutrients are not limiting. Conchosporangia production by *Porphyra dioica* requires a combination of short-days at 15ºC, without aeration, and the release of conchospores is promoted by a period of time under very low light. Aeration is also crucial for the germination of the conchospores and production of blades.

One of the key factors for the success of *Porphyra* cultivation is the establishment of a constant and readily available supply of "seedstock" of juvenile organisms (Yarish *et al.* 1998, 1999). The simplicity of requirements for the conchocelis growth and conchospore production makes *P. dioica* a good candidate for cultivation. Some species require more specific conditions and/or a specific chain of events, in terms of temperature and photoperiod, to complete their life cycles, increasing the complexity and cost of operation. Working at a commercial scale, the simplicity of the processes and low costs are important factors. The possibility of a constant natural renewal of the gametophytes can allow several harvests without the intervention of the conchocelis phase. This characteristic, if confirmed by our ongoing work, is also important for aquaculture purposes. The best conditions for the growth of the gametophytes and their possible applications (pigments, amino-acids and assimilation of nutrients) are now under investigation.

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CHAPTER 3

The influence of stocking density, light and temperature on the growth, production and nutrient removal capacity of *Porphyra dioica* (Bangiales, Rhodophyta) from Portugal

(Submitted to Aquaculture)

The influence of stocking density, light and temperature on the growth, production and nutrient removal capacity of *Porphyra dioica* (Bangiales, Rhodophyta) from Portugal

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ABSTRACT

The optimal conditions for growth of *Porphyra dioica* gametophytes were investigated in the laboratory, focusing on bioremediation potential. *Porphyra dioica* is one of the most common *Porphyra* species along the northern coast of Portugal and can be found year-round. The influence of stocking density and photon flux density (PFD) on the growth, production and nutrient removal was tested. Maximum growth rates, up to 33% per day, were recorded with 0.1 g fw l^{-1} at 150 and 250 µmol photons m^{-2} s^{-1}. Growth rate decreased significantly with increasing stocking density. Productivity had an inverse trend, with more production at the higher stocking densities. At 150 µmol m^{-2} s^{-1} and with 1.5 g fw l^{-1}, 1.4 g fw l^{-1} $week^{-1}$ were produced. At this PFD, there was no significant difference in production between 0.6 to 1.5 g fw l^{-1}. Nitrogen (N) content of the seaweeds decreased with increasing stocking densities and PFDs. The maximum N removal was recorded at 150 µmol m^{-2} s^{-1}, with 1.5 g fw l^{-1} stocking density (1.67 mg N day^{-1}). However, the N removed by thalli at 50 µmol photons m^{-2} s^{-1} was statistically equal to that at 150 and 250 µmol photons m^{-2} s^{-1}, at a stocking density of 1.0 g fw l^{-1}. The influence of temperature and photoperiod on growth and reproduction was also assessed. Growth rates of *P. dioica* were significantly affected by temperature and photoperiod. The highest growth rate, 27.54% fw day^{-1}, was recorded at 15ºC and $16:\overline{8}$ h, L:$\overline{D}$ cycle. Male thalli started to release spermatia 21 days after the beginning of the experiment, in temperatures from 10 to 20ºC and with 10, 12 and 16 hours of day length. Unfertilized female-like thalli were observed at 10 to 20ºC, under all photoperiods tested. Growth of these thalli declined after 4 weeks. By then, formation of young bladelets in the basal portion of these thalli was observed. After 7 weeks all biomass produced was solely due to these vegetatively propagated young thalli,

growing 22.4 to 26.1% day^{-1}. The results of this study showed that *Porphyra dioica* appears to be a candidate as a nutrient scrubber in integrated aquaculture systems with finfish.

Keywords: integrated aquaculture, *Porphyra*, nutrient uptake, nitrogen content, bioremediation

INTRODUCTION

Porphyra dioica Brodie *et* Irvine is one of at least 140 species described worldwide (Yoshida *et al.*, 1997; Silva, 1999). In Portugal is one of at least 5 species described (Ardré, 1970; South & Titley, 1986; Brodie & Irvine, 1997) and the most common in the North of Portugal, together with *P. umbilicalis* (L.) Kützing. *Porphyra dioica* inhabits the intertidal zone of rocky beaches throughout the year, with higher densities in late winter and spring months (Pereira *et al.*, accepted). The life cycle of *P. dioica* is biphasic and heteromorphic, with a foliose haploid gametophyte and a filamentous diploid sporophyte. The sporophyte phase it is commonly referred as the conchocelis phase (Drew, 1954). *Porphyra* is one of the world's most important maricultured seaweeds (FAO, 2001). Cultivation is exclusively done in China, Japan and Korea. Its commercial value was estimated, in 2001, at 1.2 billion US$ (FAO 2001). Despite this economic importance, all production relies in the few species being used in those countries, mainly *P. yezoensis*, *P. tenera* and *P. haitanensis* (Oohusa, 1993; Fei *et al.*, 1998; Kito & Kawamura, 1999). Besides being used for direct human

consumption, *Porphyra* can also be used as a source of r-phycoerythrin, a red pigment used as a dye in immunofluorescence reactions (Mumford & Miura, 1988; Fleurence, 1999). Due to its high surface/volume ratio, *Porphyra* is a fast growing species, capable of rapid assimilation of nutrients, namely nitrogen and phosphorus (Neori *et al.*, 2004). This, together with the mentioned economic value, makes this genus one of the most promising for bioremediation purposes and integrated aquaculture (Chopin *et al.*, 1999; Chopin *et al.*, 2001; Kraemer & Yarish, 1999; McVey *et al.*, 2002; Carmona *et* al., 2004).

In the last decade, there is a growing interest of western countries in this genus. Until now, most studies have been conducted mainly to describe the life cycles of different species, Bird (1973), Candia *et al.* (1999), Avila *et al.* (1986), Sidirelli-Wolff (1992), Waaland *et al.* (1987) and Stekoll *et al.* (1999) are just a few examples. More recently, a few studies have been dealing with physiological aspects focused on the macroscopic gametophytes (Figueroa *et al.*, 1995; Hafting, 1999a; Kraemer & Yarish, 1999; Katz *et al.*, 2000, Conitz *et al.*, 2001; Orfanidis, 2001). Simultaneously, recent molecular studies have reported on the wide cryptic molecular variation in the genus (Broom *et al.*, 2002; Klein *et al.*, 2003; Neefus *et al.*, 2002). In the United States, after an unsuccessful attempt to introduce *Porphyra yezoensis*, a research program is now being focused on the "domestication" of native Northeastern American species by Yarish *et al.* (1998, 1999, 2001).

The objective of this study is to focus on factors that are relevant in the development of the algal component of an integrated aquaculture system along the Portuguese Coast, *i.e.*: purposes, stocking densities, photon flux densities, temperatures and photoperiod. Simultaneously, we were also interested in understanding how those

factors influence the quality of the biomass and the reproduction of the gametophytes of *Porphyra dioica*.

MATERIAL AND METHODS

Stocking density, light and water analysis

The conchocelis cultures were isolated and induced to form gametophytes at 15ºC and 8-16h, L:$\overline{D}$ cycle, as described in our previous paper (Pereira *et al.* accepted). All the thalli were originated from conchocelis culture PD2-1, a strain cloned from a single zygotospore and maintained in culture at the Marine Biotechnology Laboratory, University of Connecticut at Stamford, USA. The blades used in the experiment were between 1 and 3 cm long and 2 weeks old. The experiment was carried out at 15 ºC and neutral day (12:$\overline{12}$h, L:$\overline{D}$ cycle). A combination of 5 stocking densities, 0.1, 0.3, 0.6, 1.0 and 1.5 g fresh weight l^{-1} and 3 photon flux densities (PFD), 50, 150 and 250 µmol photons m^{-2} s^{-1} were tested. Three replicates per condition were used. Blades were grown in 1 l flasks with Von Stoch's modified enriched seawater (VSE) (Ott, 1965). The medium was gently aerated. The culture medium had approximately 500 µM NO_3^-, as source of nitrogen. Twice a week, at 3 and 4 day intervals, the medium was renewed, biomass was recorded, as fresh weight (fw), and the density was reduced back to the initial stocking density. To determine the fresh weight the algae were blotted dried before weighing. The excess material was kept for tissue analysis. Samples from the incubated mediums were analyzed for inorganic N and P by the Environmental

Research Institute, University of Connecticut, using a Four Channel Auto Analyzer equipped with High-Sensitivity Seawater Cartridges (Lachat - QuikChem AE Ion Analyzer).

Samples for dry weight (dw) were taken prior to the experiments and at the end. Dry weight was measured after drying the samples for 48 h at 50°C. Dried samples were ground by using an automatic grinder (Model MM200, Retsch, Haan, Germany) and total C and N content were determined in triplicates for each replicate sample, with a Perkin-Elmer Series II 2400 CHNS/O Analyzer, Wellesley, MA. For pigment analysis the algae were immediately frozen. All the thalli were previously acclimated for 1 week to the temperature, photoperiod and different PFDs used. The experiment lasted 3 weeks.

Temperature and photoperiod

A combination of 2 temperatures 10, 15 and four photoperiods, 8-$\overline{16}$, 10-$\overline{14}$, 12-$\overline{12}$ and 16-$\overline{8}$ h, L:$\overline{D}$ cycles, were tested. Additionally, two extreme temperatures, 5 and 20ºC were tested with a 12-$\overline{12}$ h L:$\overline{D}$ cycle. Three replicates were used in all the conditions. Blades were grown in 1l flasks with VSE, as described in the previous experiment. A stocking density of 0.3 g fw l^{-1} and a PFD of 150 µmol photons m^{-2} s^{-1} were used in all the conditions. The medium was renewed twice each week. Biomass was recorded, as fresh weight and the density was reduced back to the initial stocking density. All the thalli were previously acclimated, during 1 week, to 150 µmol m^{-2} s^{-1} and to the different temperatures and photoperiods used for the experiment.

Pigment analysis

The material collected during the experiments was kept frozen at –20ºC. The analysis of phycobiliproteins (phycoerythrin and phycocyanin) concentrations was performed in aqueous crude extracts, following the method described by Beer and Eshel (1985) with some modifications. Samples of 0.03 to 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 in the cold, in dim light, and the extracts were kept in the dark at 4ºC overnight. The extracts were centrifuged at 10,000G for 20 min and the supernatant used for phycobiliprotein (PBP) determinations (phycoerythrin and phycocyanin). Light absorption was measured using a Perkin Elmer, spectrophotometer (UV/VIS spectrophotometer Lambda 20, Perkin Elmer Analytical Division of EG & G, Wellesley, MA). Concentrations of phycoerythrin (PE) and phycocyanin (PC) were calculated using the formulas described by Beer and Eshel (1985).

For Chl a analysis we followed a procedure adapted from Lobban and Chapman (1988). The tissue pellet (collected from the PBP extraction) was analysed by the following procedure. A few milligrams of $MgCO_3$ were added and the material was ground in 4ml of 90% acetone. After centrifuging for 20 min. (at 10,000 G) the supernatant was collected and the pellet was used to repeat the extraction procedure. The two extraction volumes were combined and the absorbance at 665 nm was measured using a Perkin Elmer spectrophotometer (UV/VIS spectrophotometer Lambda 20, Perkin Elmer Analytical Division of EG & G, Wellesley, MA).

Statistical analysis

For all the treatments, three independent replicates were analyzed, and means and standard deviations were calculated. For each species, differences among treatments were tested for significance using two-way ANOVA. Multiple post-hoc comparisons among means were tested by the SNK test. Data that did not comply with normality or equal variance were transformed (log x or $x^{1/2}$). In all cases, the null hypothesis was rejected at the 5% significance level, according to Sokal and Rohlf (1995),

RESULTS

Photon flux density and stocking density experiment

The gametophytes of *Porphyra dioica* grow faster at lower stocking densities and higher PFD (Fig. 1). The highest mean growth rate, over 3 weeks, was 33.6% fw day^{-1}, recorded at 250 μmol photons m^{-2} s^{-1} and 0.1 g fw l^{-1} stocking density. This value was very close to the one obtained at 150 μmol photons m^{-2} s^{-1} and 0.1 g fw l^{-1} stocking density, 32.9% fw day^{-1}. The ANOVA showed significant differences between stocking densities and between PFD ($P<0.001$) as well as an interaction between the two factors ($P<0.001$).

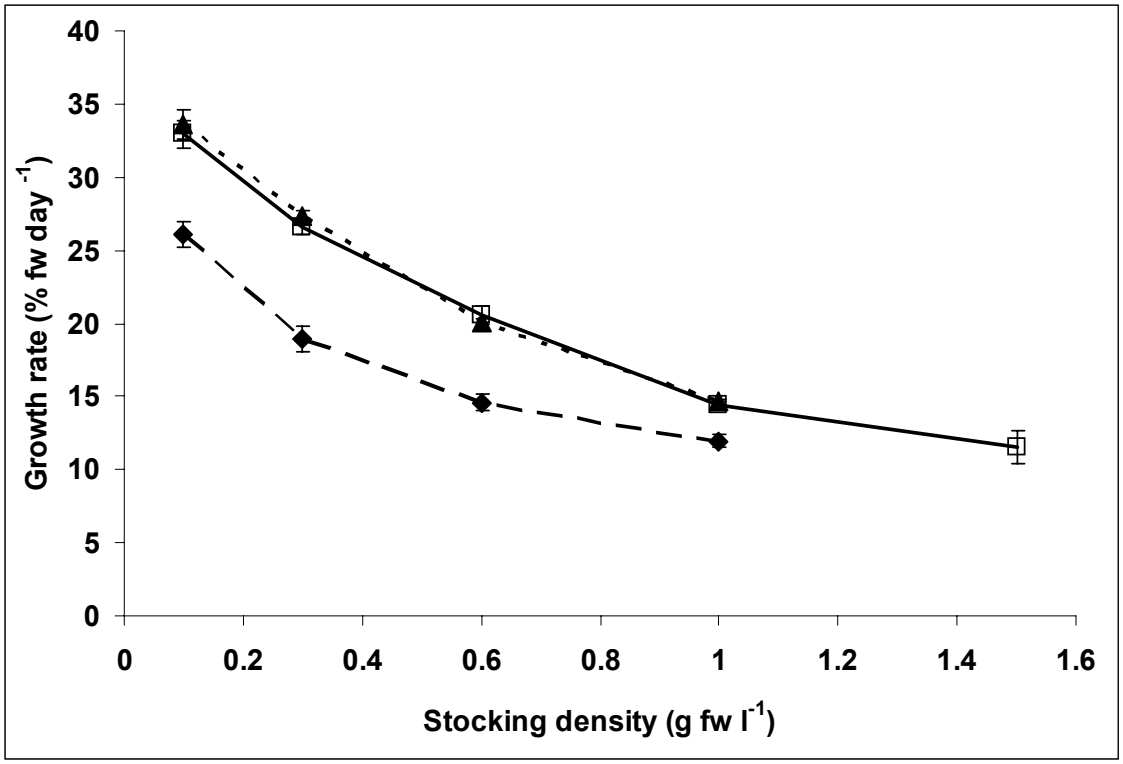

Figure 1: Effects of stocking density and photon flux density, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1} on the growth rate of the gametophytes of *Porphyra dioica* at 15ºC and 12:$\overline{12}$h, L:$\overline{D}$ cycle.

In all stocking densities the Student Newman Keuls (SNK) test showed significant differences between 50 and 150 and 250 µmol photons m^{-2} s^{-1} ($P<0.01$). No significant differences between the two highest light levels were detected. On the other hand, the SNK also showed that, within each light level, all the stocking densities are significantly different from each other ($P<0.01$).

If we consider the results in terms of productivity, the shape of the curve is inverted and the highest biomass production was obtained with the higher stocking densities and higher PFDs (Fig. 2). A stocking density of 1.5g fw l^{-1} yielded, on average, 1.40 g fw per week, at 150 µmol m^{-2} s^{-1}. A stocking density of 1.0 g fw l^{-1}, at 250 µmol m^{-2} s^{-1}, yielded 1.35 g fw per week. The ANOVA indicated that the differences between stocking density and PFD were significant ($P<0.001$). There was also a significant interaction between the two factors ($P<0.001$).

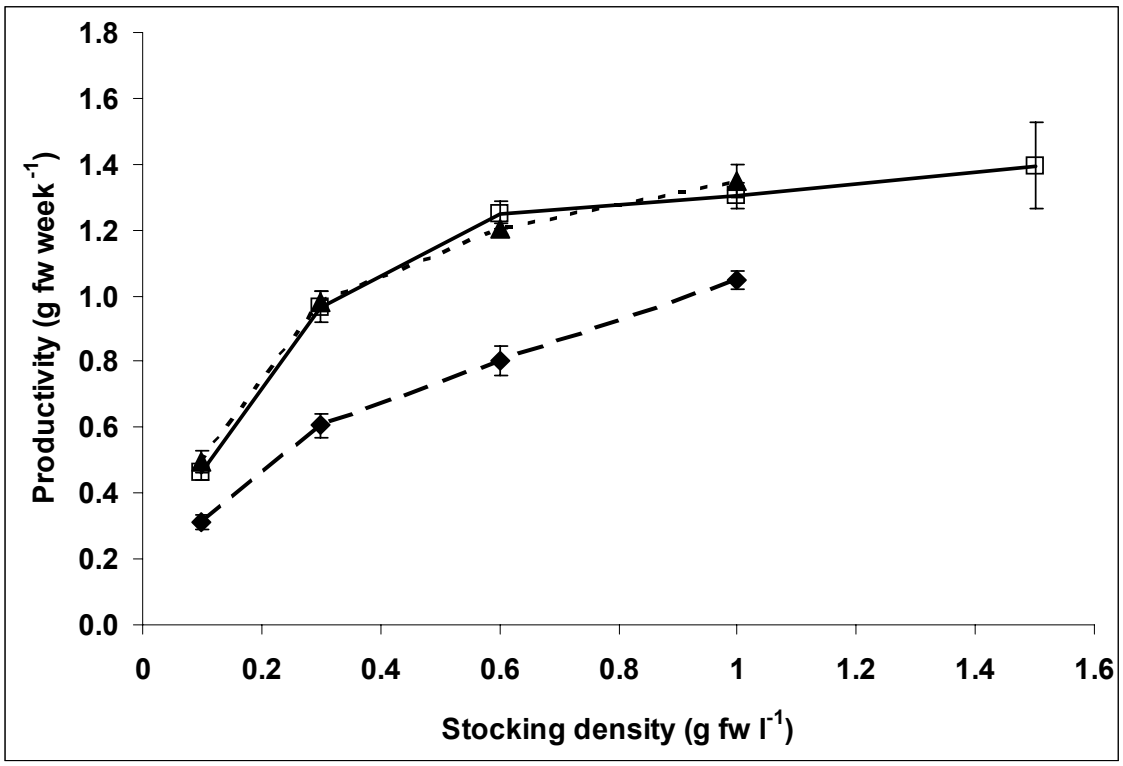

Figure 2. Effects of stocking density and photon flux density, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1} on the productivity of the gametophytes of *Porphyra dioica* at 15ºC and 12:$\overline{12}$ h, L:$\overline{D}$ cycle.

The SNK showed that, in all stocking densities, the lower productivity at 50 µmol photons m^{-2} s^{-1} was significantly different from that at 150 and 250 µmol photons m^{-2} s^{-1} ($P<0.01$). Again, as seen for growth rate, there was no significant difference between the two highest PFDs. Within each light level, the results of the SNK test are different from those observed for growth rate. At 50 µmol photons m^{-2} s^{-1}, the productivity increased significantly ($P<0.01$) with the increase of the stocking density. The same was observed for 250 µmol photons m^{-2} s^{-1}, although the productivity seemed to stabilize between 0.6 and 1.0 g fw l^{-1} of stocking density. At 150 µmol photons m^{-2} s^{-1} the productivity curve also reached a maximum after 0.6 g fw l^{-1} of stocking density. In fact, at 150 µmol photons m^{-2} s^{-1}, there was no significant difference ($P>0.05$)

between the productivity at 0.6, 1.0 and 1.5 g fw l^{-1} of stocking density. The differences between these and the lower stocking densities were significant ($P<0.01$).

The pH of the culture medium increased, as expected, after the presence of the thalli. This variation was highest at 150 and 250 µmol photons m^{-2} s^{-1}, and at higher stocking densities (Fig. 3). There was no control of the pH in the cultures during this work. CO_2 was provided by the continuous aeration of the flasks with normal air.

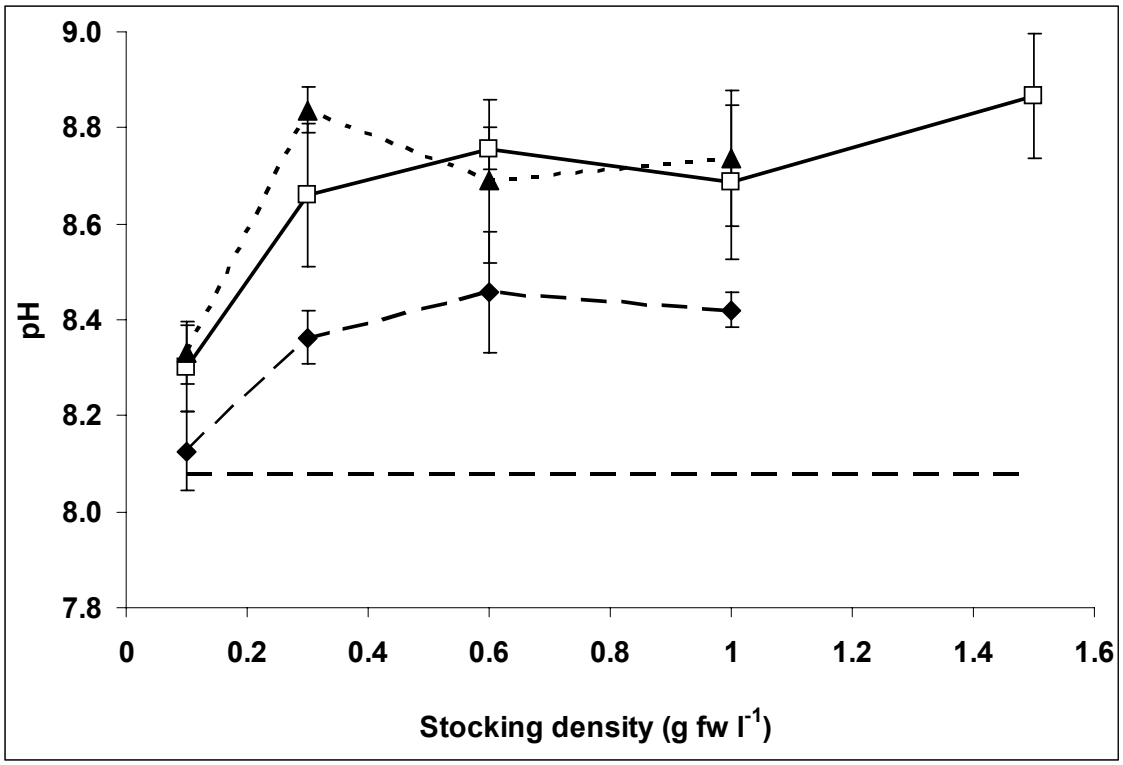

Figure 3: Variation of the pH of the culture medium, after 4 days with gametophytes of *Porphyra dioica* grown at different stocking densities and PFDs, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1}, 15º C and 12:$\overline{12}$h, L:$\overline{D}$ cycle. The horizontal dashed line closer to the x axis represents the initial pH.

The nitrogen content decreased at all PFDs tested, at increased stocking densities (Fig. 4). The nitrogen content was always higher at 50 µmol photons m^{-2} s^{-1}. The highest nitrogen percentage, 6.67% dry weight (dw), was recorded at 50 µmol photons

m^{-2} s^{-1} and 0.1 g fw l^{-1} of stocking density. The lowest, 4.24% dw, was recorded at 250 µmol photons m^{-2} s^{-1} and 1.0 g fw l^{-1} of stocking density.

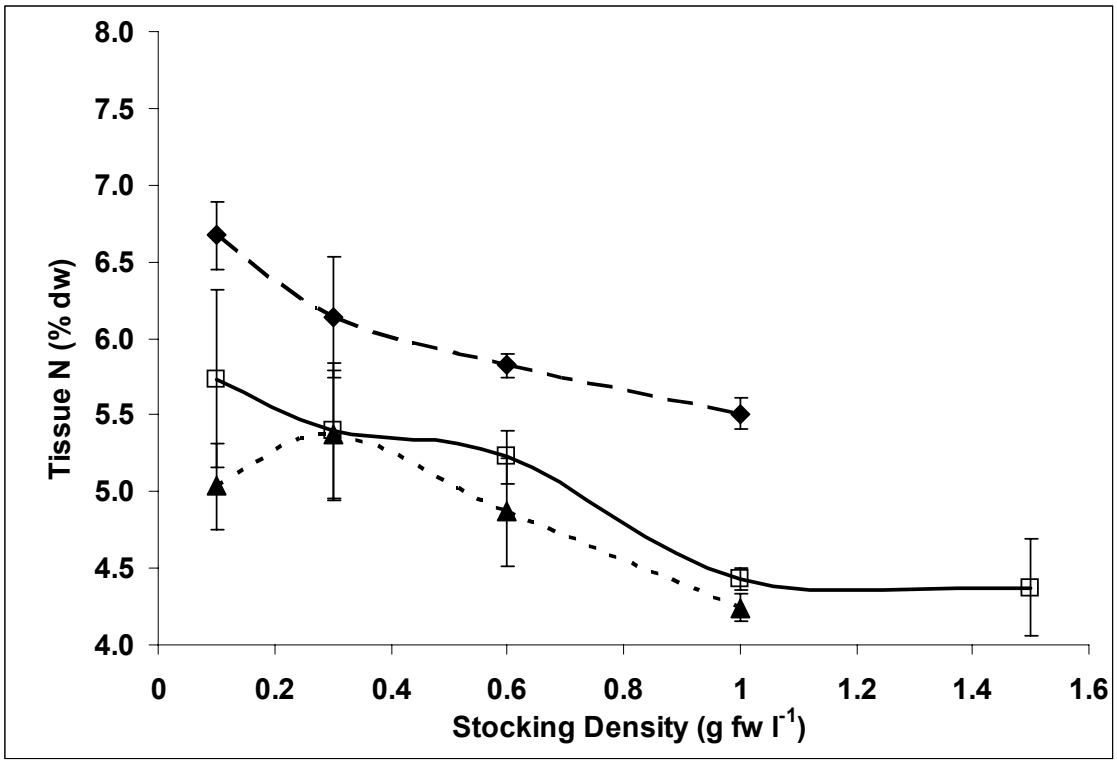

Figure 4. Effects of stocking density and photon flux density, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1} on the nitrogen content of the gametophytes of *Porphyra dioica* at 15ºC and 12:$\overline{12}$ h, L:$\overline{D}$ cycle, with 500 µM NO_3^- as source of nitrogen.

Combining the results of growth rate with the nitrogen content we also estimated the potential nitrogen removal, in mg of nitrogen per day (mg N day^{-1}), of the thalli grown in the different conditions (Fig. 5). The potential nitrogen removal increased significantly ($P<0.01$) with the increasing stocking densities, in all PFDs tested (Fig. 5).

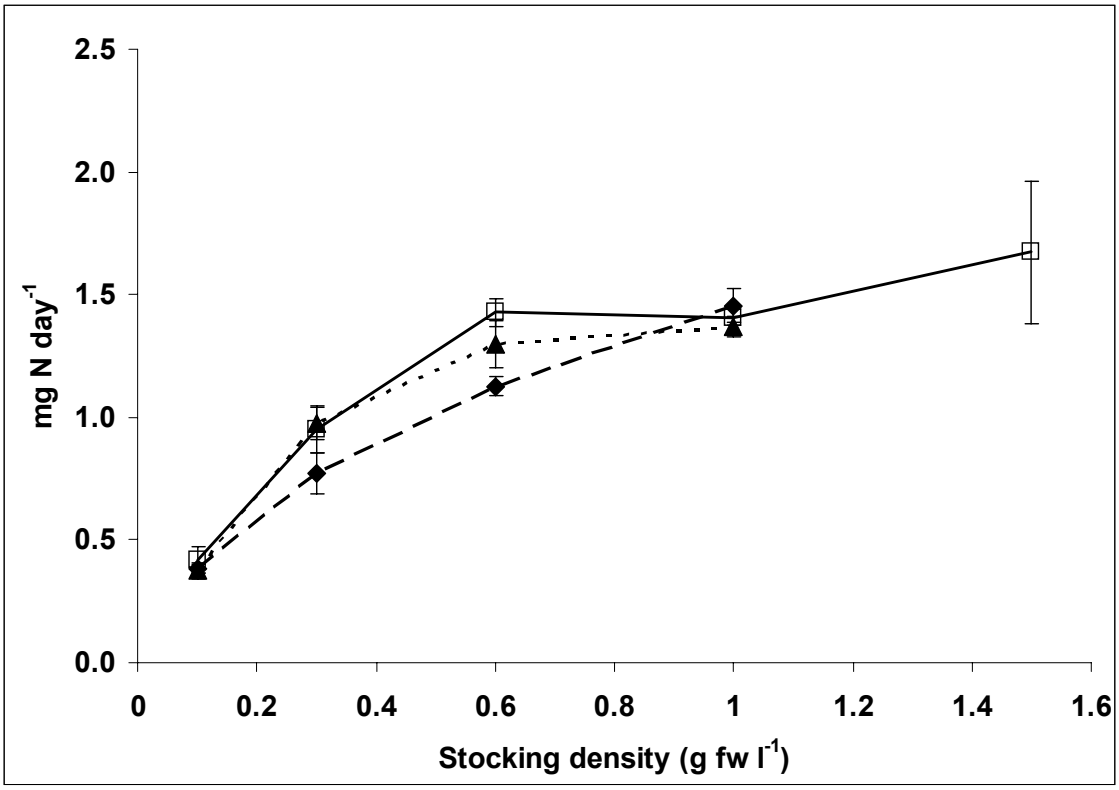

Figure 5: Potential nitrogen removal by the gamethophytes of *Porphyra dioica* grown at different stocking densities and PFDs, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1}, at 15ºC and 12:$\overline{12}$ h, L:$\overline{D}$ cycle, with 500 µM NO_3^- as source of nitrogen.

The higher nitrogen removal, 1.67 mg N day^{-1}, was achieved at 150 µmol photons m^{-2} s^{-1} with 1.5 g fw l^{-1} of stocking density. At 50 µmol photons m^{-2} s^{-1} the increase in nitrogen removed was significantly different between each stocking density. At 150 and 250 µmol photons m^{-2} s^{-1} the potential nitrogen removal increased significantly from 0.1 to 0.3 and to 0.6 g fw l^{-1}. Above the 0.6 g fw l^{-1} of stocking density the nitrogen removed was statistically equal ($P>0.05$) to that at 1.0 and even at 1.5 g fw l^{-1}. The highest stocking density was tested only at 150 µmol photons m^{-2} s^{-1}. Nitrogen removed by thalli at 50 µmol photons m^{-2} s^{-1} was statistically equal to that at 150 and 250 µmol photons m^{-2} s^{-1}, when a stocking density of 1.0 g fw l^{-1} was used.

The carbon content of the gametophytes increased, slowly but consistently, with the increasing stocking density only at 50 µmol photons m^{-2} s^{-1}, from 35.3 to 37.7% dw. At 150 and 250 µmol photons m^{-2} s^{-1} there was no defined trend. The carbon content

was always higher, varying from 38 to 39.9% dw, at the two highest PFDs than at 50 µmol photons m^{-2} s^{-1}. On the other hand, the carbon removal by the gametophytes was significantly influenced by stocking density and the photon flux density. Within each PFD, carbon removal increased significantly ($P<0.05$) with an increase in stocking density (Fig. 6).

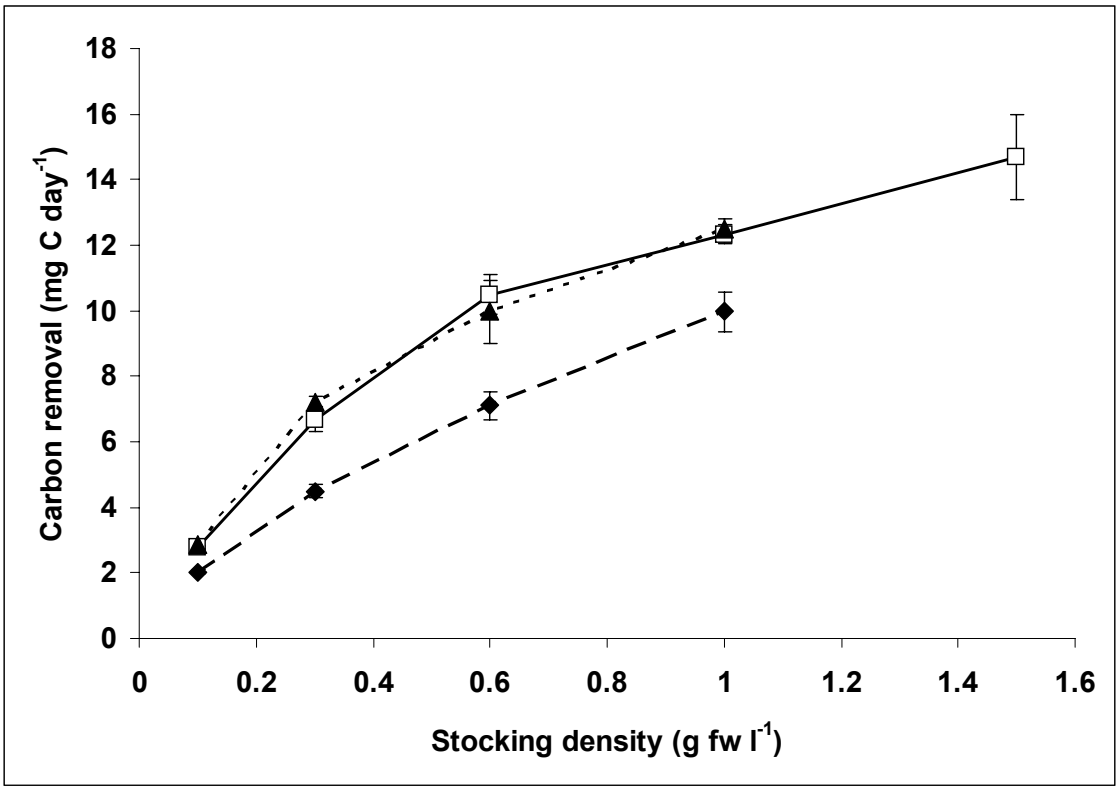

Figure 6: Influence of stocking density and PFD on the carbon content of the gametophytes of *Porphyra dioica*. (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1}, at 15ºC and 12:$\overline{12}$h, L:$\overline{D}$ cycle.

The highest value, 14.7 mg C day^{-1}, was recorded at 250 µmol photons m^{-2} s^{-1} and 1.5 g fw l^{-1} stocking density. The comparison within each stocking density showed that 150 and 250 µmol photons m^{-2} s^{-1} promoted significantly higher removal of carbon ($P<0.05$). There was no difference between these two PFDs.

The highest percentages of nitrate removed from the medium were recorded at the two highest PFDs, for all stocking densities (Fig, 7). The nitrate available in the culture medium was completely removed (*i.e.* 99.9%) in the flasks with 1.0 g fw l^{-1} or higher stocking densities at 150 µmol photons m^{-2} s^{-1}. At 0.6 g fw l^{-1} the percentage of NO_3^- removed from the medium was also higher than 99.9% at 250 µmol photons m^{-2} s^{-1} and around 96.5% at 150 µmol photons m^{-2} s^{-1}. At 0.3 g fw l^{-1}, 97.0 and 96.1% of the available nitrate was removed at 150 and 250 µmol photons m^{-2} s^{-1}, respectively. At the lowest PFD (50 µmol photons m^{-2} s^{-1}) the percentage of nitrate removed ranged from 30.2% to 88.0%, with 0.1 and 1.0 g fw l^{-1} stocking densities, respectively.

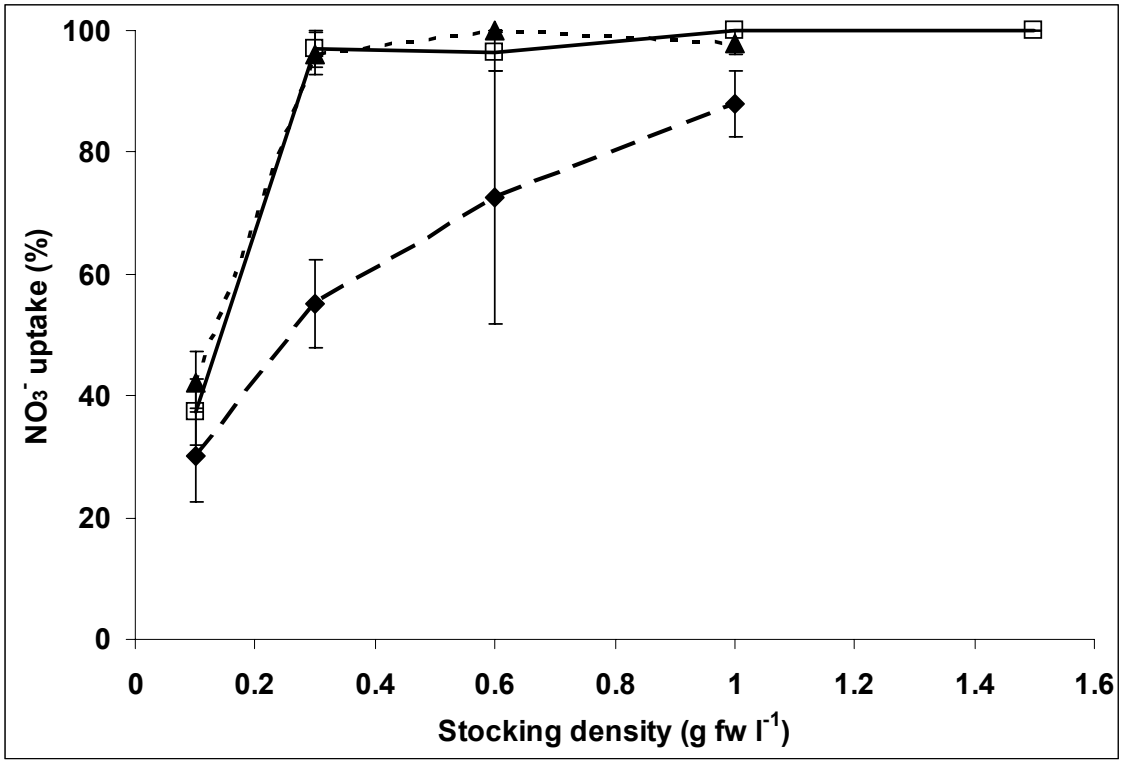

Figure 7: Uptake percentage of NO_3^- from the culture medium, after 4 days, by gametophytes of *Porphyra dioica*. Influence of stocking density and PFD. (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1}, at 15ºC and 12:$\overline{12}$h, L:$\overline{D}$ cycle, with 500 µM NO_3^- as source of nitrogen.

The phosphate uptake showed a similar pattern with the difference that the percentage uptake was not so high. The highest uptake percentage, 97.7% was recorded at 1.5g fw l^{-1} and at 150 µmol photons m^{-2} s^{-1}. At 0.3 g fw l^{-1} the percentage was around 87% for the higher PFDs.

Not all the nitrogen removed from the water was incorporated into new tissue (Fig. 8). The higher percentages of N incorporation, around 95%, were observed in the cultures with 1.5 g fw l^{-1} and 1.0 g fw l^{-1} stocking density, at 150 and 50 µmol m^{-2} s^{-1}, respectively (Fig. 8). In the cultures with 0.6 at the higher PFDs and with 1.0 g fw l^{-1} (all PFDs), approximately 400 µmol of nitrogen were incorporated in new tissue. With 0.3 g fw l^{-1}, also at the higher PFDs, around 275 µmol of nitrogen were incorporated in new tissue. At the lower PFD, 50 µmol photons m^{-2} s-^{1}, the amount of nitrogen incorporated into new tissue was smaller except for the cultures with 1.0 g fw l^{-1}. In these conditions, although the thalli removed 88% of the available N (Fig. 7), they incorporated 95% of that amount (Fig. 8). For all stocking densities, the amount of nitrogen incorporated into new tissue was closer to the amount removed from the medium at 50 µmol photons m^{-2} s^{-1} than at the higher PFDs.

Except for the thalli in cultures with 0.6 mg fw l^{-1}, the PBP content was always higher in the lower PFD (Fig. 9). The percentages of PE and PC in the total of PBP remained considerably stable, between 60 and 70% PE and between 30 and 40% PC. The average percentages, of all the conditions, were 64.1% and 35.9% (±4.67) for PE and PC, respectively. Nonetheless, for all stocking densities, the percentage of PE was always higher at the lowest PFD.

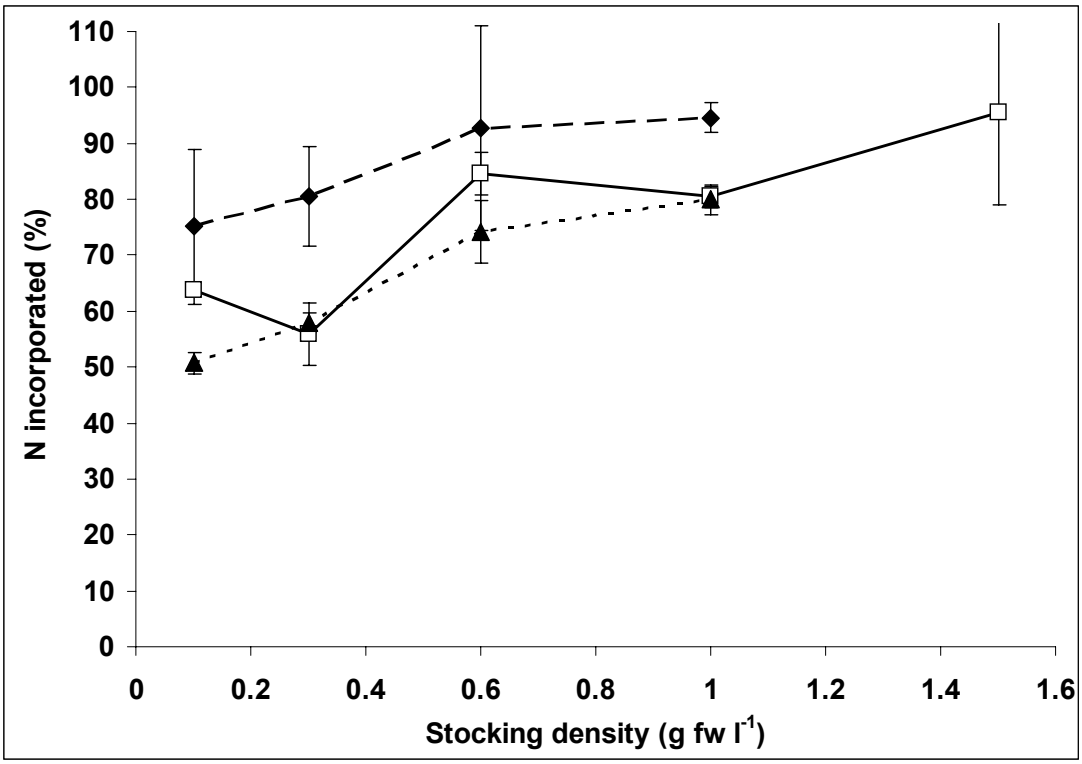

Figure 8: Percentage of N removed from the water that was incorporated into new tissue by gametophytes of *Porphyra dioica* at different stocking densities and PFDs, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1}, at 15ºC and 12:$\overline{12}$h, L:$\overline{D}$ cycle, with 500 µM NO_3^- as source of nitrogen.

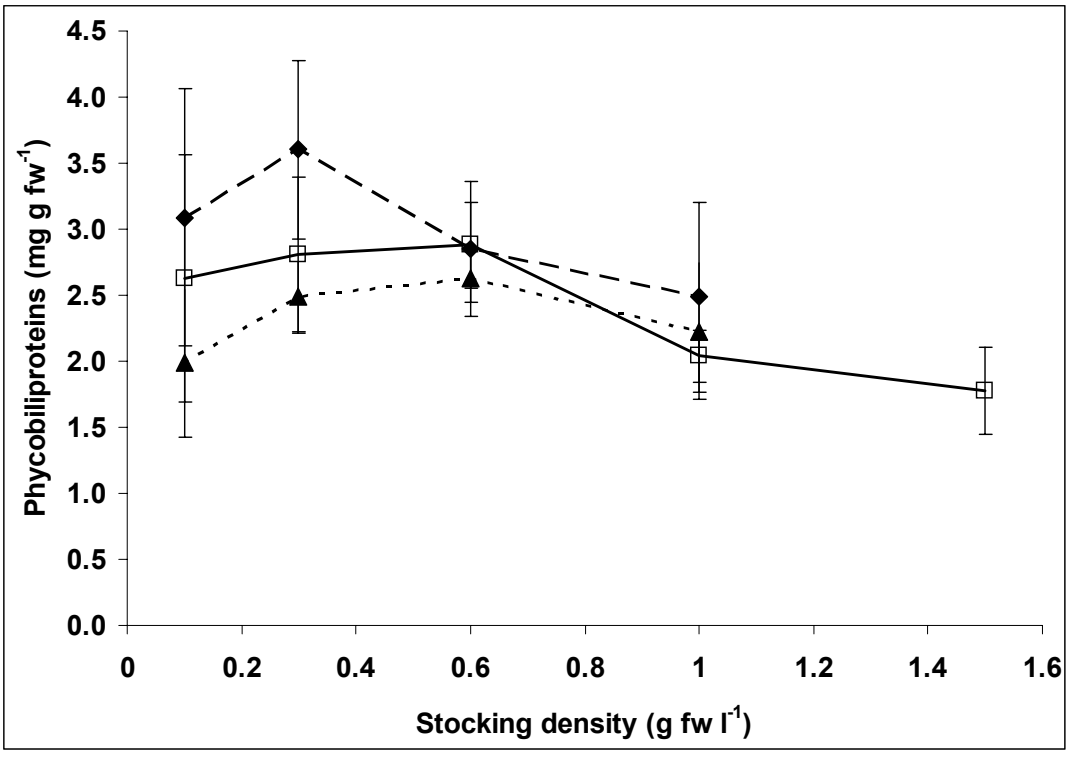

Figure 9: Phycobiliprotein content of gamethophytes of *Porphyra dioica* at different stocking densities and photon flux densities, (♦) 50, (□) 150 and (▲) 250 µmol photons m^{-2} s^{-1}, at 15ºC and 12:$\overline{12}$h, L:$\overline{D}$ cycle, with 500 µM NO_3^- as source of nitrogen.

The PE content of the thalli was significantly influenced by PFD ($P<0.01$). On average, the thalli at 50 μmol photons m^{-2} s^{-1} had 2.1 mg PE g fw^{-1}, while those at 150 and 250 μmol photons m^{-2} s^{-1} had 1.6 and 1.4 mg PE g fw^{-1}, respectively. The SNK test showed significantly higher PE content in the thalli at 50 μmol photons m^{-2} s^{-1} and no difference between the two higher light levels. There were no significant differences between stocking densities and no interaction between the two factors.

Temperature and photoperiod experiment

The objective was also to assess the influence of the photoperiod on the reproduction. In previous experiments, at 15ºC and $12:\overline{12}$ h L:$\overline{D}$ cycle, we observed male thalli releasing spermatangia 14 to 17 days after the beginning of the experiment. Some of these thalli were less than 10 cm long. This was not, however, a generalized event. After 25 days in culture there were still some male-like thalli that were not releasing spermatia and grew to 30 cm long. These results were confirmed by this experiment. Fourteen days after the beginning of the experiment (21 considering the acclimation week) we observed male thalli at 10, 15 and 20 ºC and at $10:\overline{14}$ h, $12:\overline{12}$ h and $16:\overline{8}$ h, L:$\overline{D}$ cycles. The male thalli were excluded whenever detected. The idea was to verify for how long the thalli would keep growing if there was no fertilization. Female-like thalli were first observed 18 days after the beginning of the experiment. Female-like thalli were so called because they presented the typical brick-red margins but were not releasing zygotospores. In fact, when observed under the microscope, the marginal zones were not even clearly differentiated (Fig 10A). These thalli were observed at 10, 15 and 20ºC and in all photoperiods. The female-like thalli never

released any kind of spores. After the fourth week we noticed that these female-like thalli had a thicker texture and did not look healthy. Besides, in flasks with only these thalli, growth rate decreased.

Another very interesting result was the observation of young blades forming in the basal parts of old thalli. This was first noticed after the fourth week, in all photoperiods, at 10ºC and 15 ºC but also at 20ºC, 12:$\overline{12}$ h, L:$\overline{D}$ cycle (Fig. 10B). These young thalli were responsible for growth rates obtained after the 7th week. By then almost all initial thalli had been excluded, either because they were male thalli releasing spores, or because they were old female-like thalli, not growing much or with negative growth. At 15ºC, weeks 7 to 10, growth rates averaged from 22.4 to 26.1% fw day^{-1} without significant differences between the four photoperiods.Although the thalli kept growing at a constant level throughout the 10 weeks, we decided to use data corresponding to the average of 4 weeks in culture for the statistical analysis.

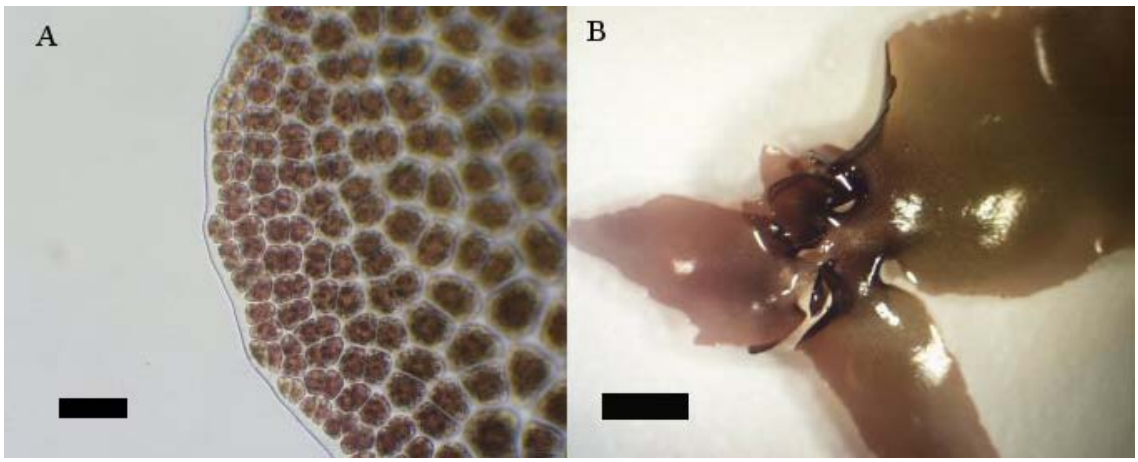

Figure 10: Blades of *Porphyra dioica* in culture. **A**. detail of the margin of a female-like thallus, there were no reproductive cells differentiated or any kind of spores released. Scale bar equals 25 μm. **B**. bladelets forming in the basal parts of older thalli. Scale bar equals 2.5 mm.

The analysis of variance showed that growth rate of the gametophytes of *Porphyra dioica* was significantly affected by temperature and photoperiod ($P<0.05$).

There was also a significant interaction between the two factors. The highest growth rate, 27.54% fw day^{-1} was recorded at 15ºC and 16:$\overline{8}$h, L:$\overline{D}$ cycle (Fig. 11). According with the SNK test, this value was significantly different (P<0.01) from the growth rate at 15ºC, 12:$\overline{12}$h and 10:$\overline{14}$h, L:$\overline{D}$ cycles, 25.24 and 25.17% fw day^{-1}, respectively. The SNK also showed that there was no significant difference between 12:$\overline{12}$h and 10:$\overline{14}$h, L:$\overline{D}$ cycles.

At 10ºC, growth rate at 12:$\overline{12}$h and 16:$\overline{8}$h, L:$\overline{D}$ cycles was significantly higher than that at 8:$\overline{16}$h and 10:$\overline{14}$h, L:$\overline{D}$ cycles (P<0.01). There was no significant difference within each pair.

In all photoperiods, the growth rate was significantly higher at 15ºC than at 10ºC (P<0.01).

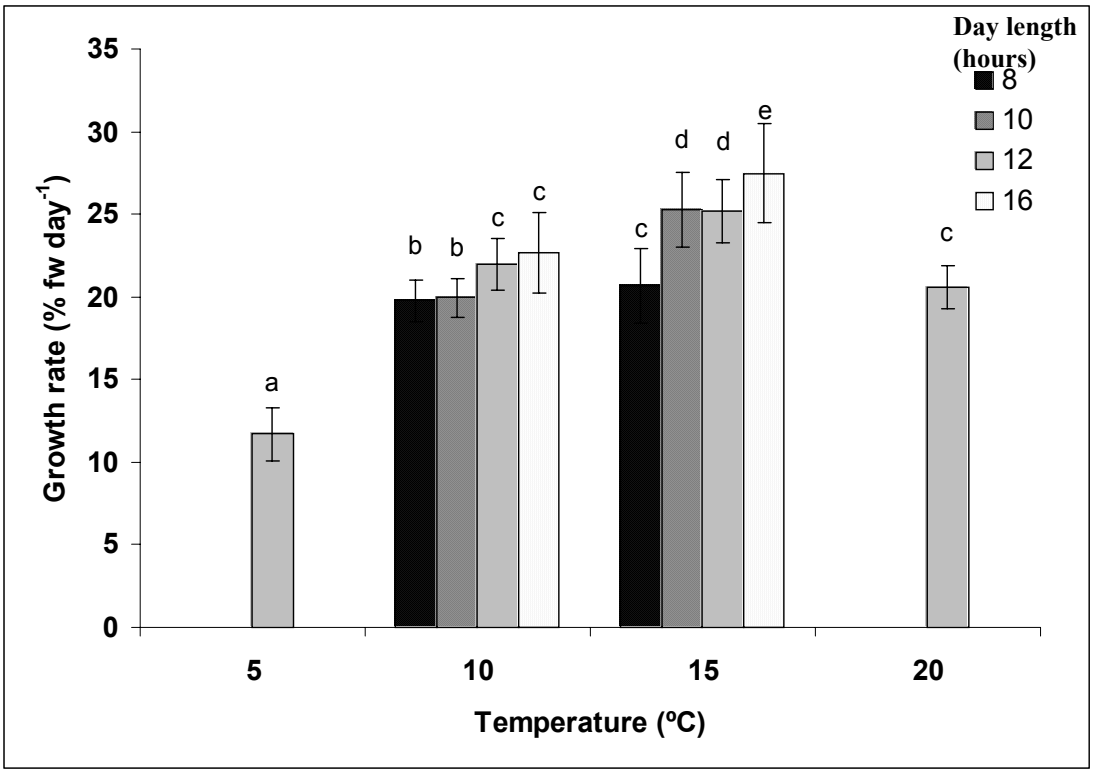

Figure 11: Growth rate of the gametophytes of *Porphyra dioica* under different temperatures and photoperiods. The legend in the figure represents day length (hours). Other conditions controlled included PFD, 150 µmol photons m^{-2} s^{-1} and stocking density, 0.3 g fw l^{-1}. Bars represent the average of 3 replicates, during 4 weeks, with the correspondent standard deviation. Different letters on top of the bars represent statistical significance between conditions.

At 12:$\overline{12}$ h, L:$\overline{D}$ cycle, where more temperatures were tested, growth rate was significantly higher at 15ºC ($P<0.01$) than in other temperatures. There was no significant difference between 10 and 20ºC, but these had, in turn, significantly higher growth rates than at 5ºC.

In terms of nitrogen in the tissue, there was no significant influence of the different photoperiods at 10 and 15ºC ($P>0.05$). There was also no significant difference between these two temperatures ($P>0.05$). The ANOVA performed with growth rate data from the different temperatures tested under 12:$\overline{12}$ h, L:$\overline{D}$ cycle, also showed that there are no differences from 5 to 20ºC. The highest content of nitrogen in the tissue was recorded for the thalli grown at 10ºC and 12:$\overline{12}$ h, L:$\overline{D}$ cycle, with 5.7% dw (Fig. 12).

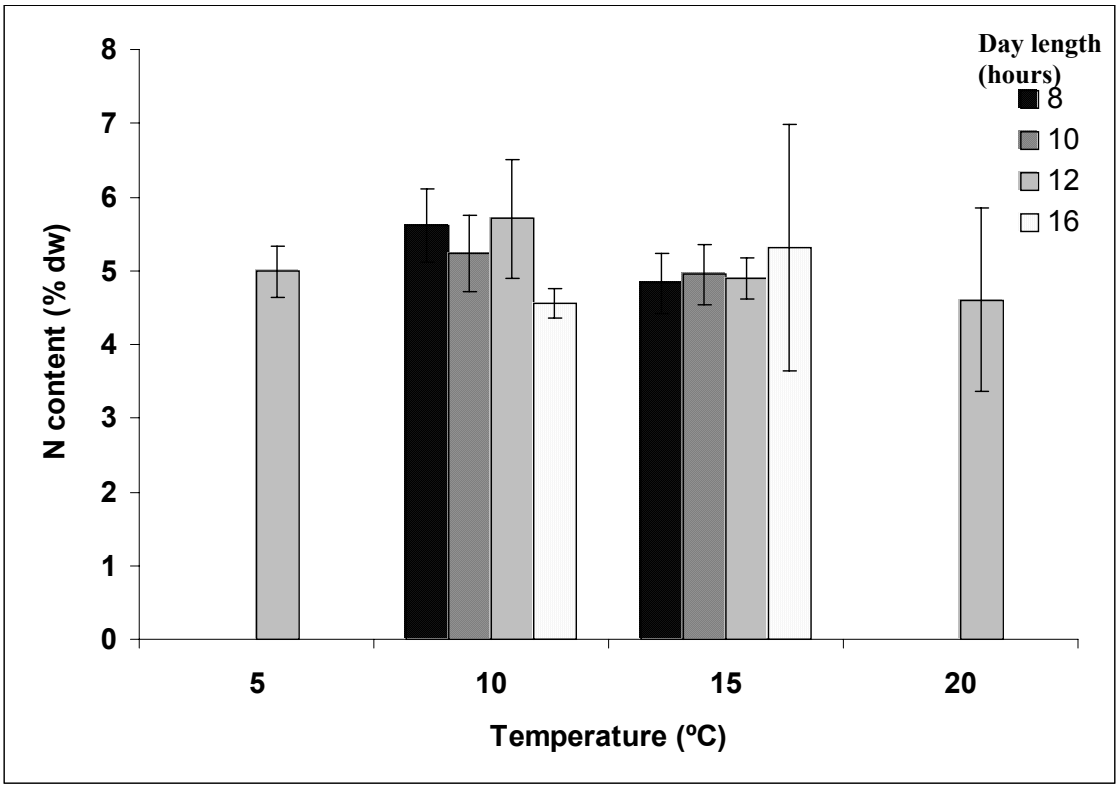

Figure 12: Nitrogen content of the gametophytes of *Porphyra dioica* under different temperatures and photoperiods. The legend in the figure represents day length (hours). Other conditions controlled included PFD, 150 µmol photons m^{-2} s^{-1} and stocking density, 0.3 g fw l^{-1}. Bars represent the average of 3 replicates, during 4 weeks, with the correspondent standard deviation.

The nitrogen removal capacity of the thalli was higher at 15ºC and 16:$\overline{8}$h, L:$\overline{D}$ cycle, with 1.12 mg N day^{-1} (Fig. 13). This value was significantly higher than those at other photoperiods and temperatures. At 10ºC, the nitrogen removal capacity was not significantly influenced by the photoperiod. Comparing the four temperatures tested in 12:$\overline{12}$h, L:$\overline{D}$ cycle, the ANOVA showed significantly lower nitrogen removal at 5ºC. There was no significant difference between 20, 15 and 10ºC.

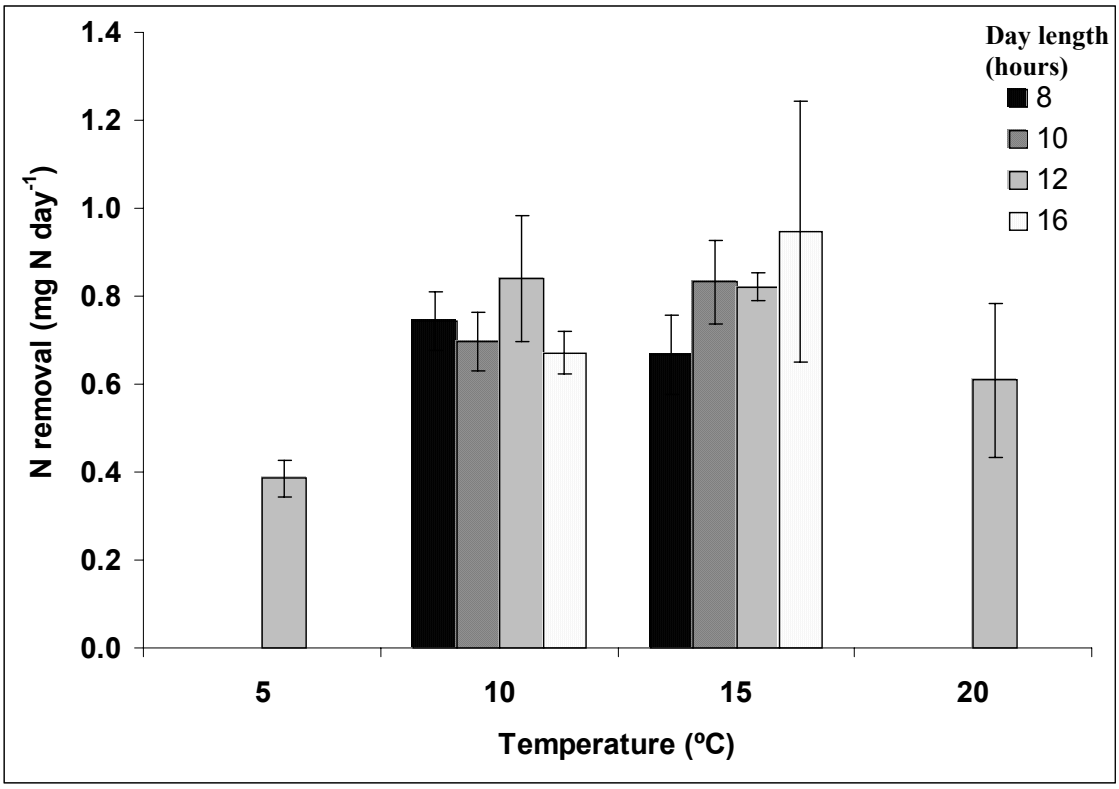

Figure 13: Nitrogen removal capacity of the gametophytes of *Porphyra dioica* grown at different temperatures and photoperiods, under 150 µmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density and 500 µM NO_3^- as source of nitrogen. Bars represent the average of 3 replicates, during 4 weeks, with the correspondent standard deviation.

The carbon content of the gametophytes of *Porphyra dioica* was higher on thalli at 20ºC and 12:$\overline{12}$h, L:$\overline{D}$ cycle, with 39.8% dw. This value was not significantly higher than that of the thalli at 10 and 5ºC and same photoperiod (Fig. 14). The carbon content of the thalli at 15ºC was significantly lower. At 15ºC the carbon content decreased

significantly with increasing day length, from 8 to 10 hours per day ($P<0.01$). Only at $8:\overline{16}$ h, L:$\overline{D}$ cycle, the carbon content was higher at 15ºC than at 10ºC.

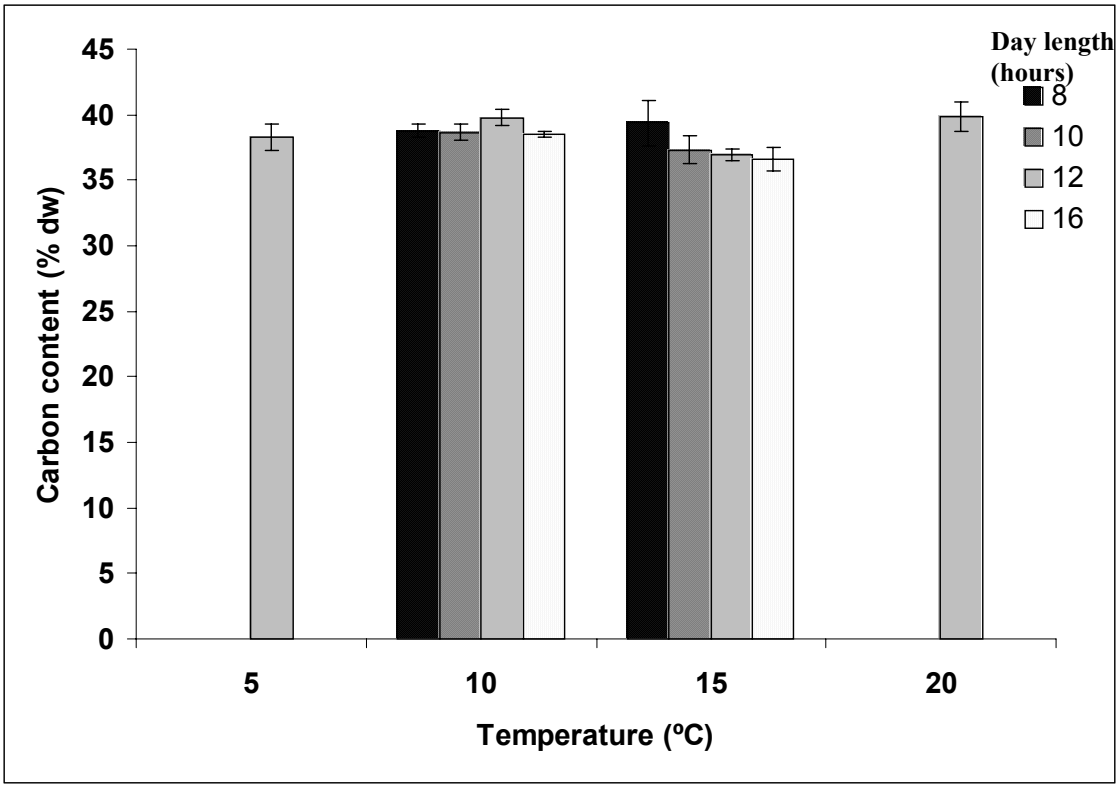

Figure 14: Carbon content of the gametophytes of *Porphyra dioica* under different temperatures and photoperiods. The legend in the figure represents day length (hours). Other conditions controlled included PFD, 150 µmol photons m^{-2} s^{-1} and stocking density, 0.3 g fw l^{-1}. Bars represent the average of 3 replicates, during 4 weeks, with the correspondent standard deviation.

The PBP and chlorophyll *a* contents were not significantly influenced by the temperatures or photoperiods tested. The highest value of PBP, 6.3 mg PBP g^{-1} fw, was recorded at 10ºC and $12:\overline{12}$ h, L:$\overline{D}$ cycle. This corresponded to 3.54 and 2.77 mg g^{-1} fw, of PE and PC, respectively. For chlorophyll *a*, the maximum, recorded at 15ºC and $16:\overline{8}$ h, L:$\overline{D}$ cycle, was 2.68 mg g^{-1} fw. At 15ºC, the phycoerythrin and phycocyanin content showed a slight tendency to decrease with an increase in day length. The Chl *a* did not follow this tendency. In terms of PE and PC fractions, there was a higher percentage of PE in thalli at 5ºC and $12:\overline{12}$ h, L:$\overline{D}$ cycle, when compared to all the others. At these conditions, PE and PC represented 74.4% and 25.6% (±1.07) of the

total PBP content, respectively. The average for all other conditions was 58.2% and 41.8% (±3.29), for PE and PC, respectively.

DISCUSSION

The best growth rate of the gametophytes of *Porphyra dioica* was obtained at lower stocking densities and 150 to 250 μmol m^{-2} s^{-1}. At these PFDs and with only 0.1 g fw l^{-1} stocking density, *P. dioica* grows over 30% day^{-1}. Being a fast grower and able to assimilate nutrients rapidly, it may be expected to have higher growth rates when nutrients are higher.

The effects of the temperature on the growth rate showed that *Porphyra dioica* is well adapted to the water temperatures in its natural habitat. The water temperature in the North of Portugal ranges roughly from 11 to 21ºC (Anonymous, 2003) and *P. dioica* is most abundant at 15ºC (Pereira *et al.* accepted).

Although the statistical analysis detected significantly higher growth at 15ºC and 16:$\overline{8}$ h, L:$\overline{D}$ cycle, the results obtained in other conditions should be noted. *Porphyra dioica* was able to achieve growth rates over 20% fw day^{-1} from 10 to 20ºC. We also confirmed that thalli have a limited life span. Males reproduce at all photoperiods and 10-20ºC after 14 days in culture (21 days considering the acclimation week or approximately 42 days considering the time since conchospore release). Female-like thalli also stopped growing, approximately after 28 days in culture, even without fertilization and loss of tissue by sporulation. The formation of young blades from the basal parts of adult thalli can explain the presence of *P. dioica* thalli throughout the year

along the North Portuguese Coast, a question mentioned in our previous communication (Pereira *et al.* accepted).

The effects of photoperiod on the growth rate were expected. We knew from previous experiments that the gametophytes were able to grow in photoperiods with 8 to 16 hours of light per day. Other authors showed that the production is closely related to the number of hours of light (Bidwell *et al.*, 1984). In *Chondrus crispus*, net productivity in tank culture is a linear function of irradiance over temperatures of 10-20ºC when nutrients are not limiting (Craigie, 1990). A similar relation was found for *Porphyra dioica* in this study, where growth rates increased with increasing day length. It is interesting to note the influence of the stocking density on biomass production. Higher yields may be obtained at higher stocking densities and higher PFDs. At these PFDs and with these stocking densities production is not light limited.

Nitrogen limitation is unlikely to occur at 500 µM NO_3^- if pulsed every 3-4 days, except at the highest stocking density. With a stocking density of 1.5 g fw l^{-1} and at 150 µmol photons m^{-2} s^{-1}, with a biomass production of 1.39 g fw per week, the thalli would remove from the water 1.67 mg N per day. This amounts to a N removal of 5.0 to 6.69 mg N per each nutrient pulse (3-4 days), corresponding to an uptake of 358 to 478 µmoles of NO_3^- (72 to 95% of the N available in the medium). At lower stocking densities the amount of N incorporated into new tissue after 4 days is lower. Comparing between stocking densities at 150 µmol photons m^{-2} s^{-1}, with 0.1, 0.3, 0.6 and 1.0 g fw l^{-1} stocking densities, 119, 271, 408 and 402 µmoles of nitrogen, respectively, were incorporated in new tissue. This corresponds, in the same order, to approximately 64, 56, 85 and 80% of the nitrogen removed from the medium (see Fig. 8). In other words, although the thalli were removing more than 92% of the available N (except the ones

with 0.1 g fw l^{-1}), they were incorporating into new tissue only a fraction of this nitrogen.

When we compare stocking densities within the other two PFDs the trend is similar. However, at 50 μmol photons m^{-2} s^{-1} the percentage of N incorporated into the tissue is always higher for all stocking densities when compared to the higher PFDs. The higher N content of these thalli agrees with these results. It seems that although the thalli could remove an enormous amount of N from the water, they were not able to metabolize all that and incorporate it into new tissue. This is a very important question if this alga is to be considered for bioremediation purposes. In this case, the uptake efficiency cannot be determined simply by water analysis and quantification of the percentage of N removed from the water.

Tyler *et al.* (1994) reported for the first time the release of dissolved organic nitrogen (DON) by *Ulva lactuca* during active growth. Release of free amino acids (FAA) was measured before in studies with phytoplankton (Collos *et al.*, 1992; Bronk & Ward, 1999). On the other hand, Naldi and Wheeler (2002), using ^{15}N isotopes, observed very little release of DON by *Ulva fenestrata* and *Gracilaria Pacifica*. Naldi and Wheeler (2002) suggested that the release of DON is more likely to be due to degradation of detritus than from live algal tissue. In the case of our study there were no detritus present in the system. What happened to that missing nitrogen fraction is a question that remains to be answered. Further studies are being planned to determine the N uptake and N incorporation kinetics and the N saturation levels for this species.

Another limiting factor could be CO_2 limitation (Craigie & Shacklock, 1995). The measurements taken revealed that the pH changes to a maximum of 8.8 after 4 days at stocking densities of 0.3 and 1.5 g fw l^{-1}. A similar change was observed in all the conditions where biomass production was higher. In other words, pH changed as much

in cultures with 1.5 g fw l^{-1} as in those with 0.6 and even 0.3 g fw l^{-1}. We do not know, at this point, if *Porphyra dioica* is capable of using HCO_3^- as source of carbon.

Bicarbonate utilization has been suggested for *Porphyra leucosticta* (Mercado *et al.*, 1997), *P. umbilicalis* (Maberly, 1990) and, although with a limited capacity, for *P. linearis* (Israel *et al.*, 1999). On the other hand, those authors also agree that species restricted to utilizing CO_2 did not increase pH above 9.0. At this pH, CO_2 levels would account for only 0.06% or less of the inorganic carbon available in the seawater (Beer and Eshel, 1983). This seemed to be the case for *P. dioica*. If *P. dioica* is not able to use bicarbonate then we must consider that CO_2 limitation could have occurred. In this case, we must also note that carbon limitation is likely to occur in stocking densities as low as 0.3 g fw l^{-1} if pH is not controlled. On the other hand, this can also mean that growth rate and, consequently, productivity can be improved adding inorganic carbon to the culture medium. Gao *et al.* (1991) reported that photosynthesis and growth rate of *P. yezoensis* was enhanced when the cultures were aerated with CO_2.

Porphyra dioica is characterized by rapid growth and nutrient assimilation, respectively. The results confirm what is predicted by the functional-form model (Hanisak *et al.*, 1990). However, like those authors also have explained, production of a species with this flat blade morphology is not sustainable for significant periods of time. Large portion of the thalli may become reproductive, entire cultures can sporulate and be lost in short time period. For this reason, one characteristic of the *Porphyra* cultivation is the need for a constant supply of zygotospores. Each crop, every year, has to start from a conchocelis culture that is induced to form conchosporangia and to release conchospores at a desired moment. In other words, there must be a whole operating system behind the profitable gametophyte culture. The whole system is

usually labor and cost intensive and this is one of the reasons why this industry has not been established in developed countries, where salaries are usually higher.

Several studies have focused on vegetative propagation techniques for *Porphyra*, bypassing the need for the sporophyte culture (*e.g.* Notoya, 1999; Hafting, 1999b; Chen, 1997). Our efforts have revealed that *P. dioica* is capable of regenerating new blades from the basal portions of adult blades. It becomes even more interesting to note that such phenomena happened in temperatures from 10-20ºC (and, at least from 10-15ºC, in all photoperiods tested). The growth rate of these new blades varied between 22.4 and 26.1% day^{-1} and was similar to the initial blades and similar in all photoperiods. The applications of these observations are easy to understand in light of what was previously said. This would, at least theoretically, allow a continuous culture of gametophytes without the need of a periodic "seeding" procedure and the maintenance of conchocelis cultures in the development of a land-based integrated recirculating system. Clonal cultures would be less costly to maintain.

Neori *et al.* (2004) have already pointed the limitations of the use of bacterial biofilters for the treatment of intensive aquaculture waters. According to those authors, it is the added cost that prevented bacterial-based intensive aquaculture technologies from producing large quantities of fish at competitive prices. Neori *et al.* (2004) also described the advantages of the use of seaweed-based biofiltration. There is not, however, a global solution. Land-based integrated aquaculture systems are dynamic, changing according to such variables as location, season, species and social environment (Edwards, 1994; Little & Muir, 1987).

The wide range of temperature and photoperiod on which *Porphyra dioica* is able to grow above 20% day^{-1} it is also an interesting feature. If this genus is acceptable for biofiltration of land-based finfish aquaculture, flexibility will be important. Using

eurythermal species provides an advantage for year round aquaculture. Other important aspects are the nutrient removal efficiency of *P. dioica* and the quality of its biomass. Recently, Troell *et al.* (2003) published a comprehensive review of the major studies done within an integrated aquaculture perspective. The results obtained for N uptake in our work compare well with ones referred by those authors. The figure of N removal is similar to the productivity figure. At 150 and 250 μmol photons m^{-2} s^{-1} the N removal data are close to each other above 0.6 g fw l^{-1} stocking density, inclusive (see Fig. 5). Again there was no difference between 150 and 250 at 0.6 and 1.0 g fw l^{-1} stocking density. The main difference is that thalli grown at 50 μmol photons m^{-2} s^{-1} have a nitrogen removal capacity similar to that of the thalli grown at higher light levels. At 0.1 and 1.0 g fw l^{-1} stocking density this capacity is actually statistically equal ($P>0.05$). These results might come as a surprise because of the significantly lower growth rates and productivity of the thalli at 50 μmol photons m^{-2} s^{-1}. The differences on growth rates are certainly balanced by their higher nitrogen content, especially at higher stocking densities. An important pool of nitrogen in the tissue are the phycobiliprotein pigments, namely PE. The PE content at the lower light level is 21.7% and 30.4% higher than at 150 and 250 μmol photons m^{-2} s^{-1}, respectively. This effect has been reported as photoacclimation (Falkoswki & LaRoche, 1991). The values obtained in this study are similar to those reported for other *Porphyra* species. Conitz *et al.* (2001) detected 1.80 to 3.67 mg g^{-1} dw in young gametophytes of *P. linearis* grown at 90-110 μmol photons m^{-2} s^{-1}, 8:$\overline{16}$h, L:$\overline{D}$ cycle, although these authors used less nitrate (88 μM). It seems, however, that the higher PE content at 50 μmol photons m^{-2} s^{-1} might not be sufficient to explain the equivalent N removal at this PFD, when compared to the N removal at 150 and 250 μmol photons m^{-2} s^{-1}, where productivity is significantly higher. Other important pools of N in the tissue are the FAA and micosporine-like

amino acids (MAA). The effects of these culture conditions on the FAA and MAA quantity and quality in *P. dioica* need to be investigated. Pedersen *et al.* (submitted) showed that FAA is positively correlated with the total N content in *P. perforata*, *P. suborbiculata* and *P. leucosticta* Type C (*sensu* Neefus *et al.*, 2000)

In conclusion, the gametophytes of *Porphyra dioica* can sustain a high growth rate and productivity over a range of temperature, photoperiod and light conditions. The optimal conditions can be adapted to the objective of the culture. Stocking density of the culture is important in terms of biomass production. In combination with light, these factors influence the biochemical properties of the thalli. If the purpose of the culture is to obtain biomass with higher N content (> 6% dw), for instance for phycoerythrin extraction, stocking densities of 0.3 and 0.1 g fw l^{-1} and 50 μmol photons m^{-2} s^{-1} should be used. If the focus is on biomass production and maximum nutrient assimilation, higher stocking densities and PFDs should be used. This difference is relevant if we think in terms of bioremediation application. Chopin *et al.* (2001) pointed that the concepts of nutrient uptake efficiency and nutrient uptake rates must be distinguished. Neori *et al.* (1996) showed a way to combine both results using seaweed biofilters integrated with intensive fish aquaculture. Their system consisted of fish tanks--seaweed tanks water recirculation system plus a polishing tank with seaweeds. Higher uptake efficiency in the polishing tank was achieved by reducing the water influx.

Trying to extrapolate the potential N-removal results obtained in this study into a bigger scale, we will consider that: approximately 600 g of N are released per day by ton of fish (data from a 72 fish pond, turbot and sea bass farm, as described by Costa *et al.*, submitted); in a 1 m^3 seaweed tank (1 m^2 surface area), we could have 1.5 kg of *Porphyra dioica* in culture, capable of removing 1.67 g N per day. The area needed to remove 50% of the N effluent would be 179 m^2 of *Porphyra dioica* culture. We believe

that at this scale the performance of *P. dioica* can be improved. Under this tank cultivation conditions, *P. dioica* would have more nutrients and CO_2 available, due to the constant water flux. This would also allow to experiment with higher stocking densities, possibly increasing nutrient removal.

The results of this study show that *Porphyra dioica* should be considered for in the bioremediation of aquaculture effluents. The growth rates obtained, the N removal potential and the possibility of vegetative propagation are promising features for land-based integrated aquaculture systems.

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CHAPTER 4

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

(Submitted)

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

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ABSTRACT

Aspects of the nutrient uptake physiology of *Porphyra dioica* collected in Porto, Portugal, were investigated under laboratory conditions. The nitrogen (N) uptake and accumulation capacity of *Porphyra dioica* were determined for two different N forms, 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 uptake from the medium and accumulate equally well both forms of nitrogen. The form of N used also did not influence the growth rate. The photosynthetic pigments increased significantly with the increase of the available N in the medium, for both N forms. The form of N available did not influence the phycobiliprotein content, whereas the chlorophyll *a* content was higher in the thalli that used nitrate as source of N. In the presence of both N forms, *P. dioica* removed preferentially ammonium, with a clear day and night influence. The algae removed 70% of the ammonium available during the day and only 35% during the dark period. The P enrichment did not influence the growth rate or the amount of P removed from the medium, suggesting lack of ability to store P. These results confirm that *P. dioica* is a good candidate for application in integrated aquaculture.

INTRODUCTION

Porphyra is one of the most important maricultured seaweeds in the world. In 2001, according with data from the Food and Agricultural Organization (FAO, 2003), 1.132 million metric tons of *Porphyra* (nori) was produced, valued at 1.2 billion USD. This value is only passed by the production of *Laminaria japonica*, with 4.683 million metric tons valued at 2.8 billion USD (FAO, 2003). Data from FAO show that production of *Porphyra*, by weight, represents 16.3% of the world's seaweed mariculture which, in turn, represents 28.4% of the world's total mariculture production including fish, aquatic plants, molluscs and crustaceans. *Porphyra* is primarily used for food but also as a source of the red pigment *r*-phycoerythrin (Mumford and Miura, 1988). The genus has, however, much more potential and can be used as an experimental system for applied and basic research (Sahoo *et al.,* 2002).

The amount of fixed, or available nitrogen (N) has increased during the last century (Seitzinger, 2002). Biological N_2-fixation was the major source of newly fixed N before 1800 and amounted to approximately 90-130 millions tons of N per year (Galloway *et al.*, 1995). This amount had doubled as of 1990 due to the production of synthetic fertilizers, increased agricultural crops, and atmospheric NO_x deposition associated with fossil fuel combustion (Galloway *et al.*, 1995). Walsh and Dieterle (1988) found that human-related loading of nitrogen have increased tenfold during the last century and is responsible for the eutrophication of coastal waters. However, nutrient-enriched coastal waters are responsible for 95% of the world's fisheries (Walsh and Dieterle, 1988). Besides fisheries harvests, coastal areas are temporary habitats for the juvenile stages of many economically and ecologically important species (Clark, 1992). The environmental consequences of the nutrient enrichment of coastal

ecosystems, *i.e.*, increased phytoplankton production with possible increases of harmful algal blooms, increased water turbidity, decrease of submerged aquatic vegetation, oxygen deficiency (hypoxia), decrease in biodiversity and alteration of food web structure are well documented (*e.g.* Nixon, 1995; Capriulo *et al.* 2002; Rabalais, 2002; Guo and Li, 2003).

Eutrophication is a process and not a trophic state (Nixon, 1995). Although the term is commonly associated with ecological problems, its meaning is simply the process of "increase in the rate of supply of organic matter to an ecosystem." The impact of the organic enrichment will depend on the rate at which the nutrients dilute before being assimilated by other organisms (Carroll *et al.* 2003). In restricted exchange environments there is a risk of high levels of nutrients accumulating in one area, causing hypernutrification and leading to many undesirable consequences (MacGarvin, 2000; Chopin and Yarish, 1999)

As recognized by the PEW Oceans Commission (Goldburg *et al.*, 2001), the contribution of aquaculture is small when compared to the largest U.S. sources of pollution, but it can be locally very significant. In the L'Etang Inlet, in New Brunswick, Canada, aquaculture is the largest anthropogenic source of nitrogen and phosphorus (Strain *et al.*, 1995; Chopin *et al.*, 2001). Although the global eutrophic impact of aquaculture is relatively minor at this time, it is increasing and must be accounted for in management strategies (McVey *et al.*, 2002; Troell *et al.*, 2003). Coastal eutrophication is a cumulative problem (Goldburg *et al.*, 2001) often caused by many unidentified sources.

Despite the problems associated with it, aquaculture activities are and will continue to be one of the most significant ways to compensate for the reduced fisheries yields that are inevitable in the future (FAO 2000, 2003). According to the Food and

Agricultural Organization of the United Nations, the total world capture marine fisheries annual production has been nearly level since 1986 (FAO, 2000, 2003). During the same period, global marine finfish and shellfish aquaculture production has increased nearly 10% per year, making aquaculture the fastest growing global food production sector (Chopin *et al.*, 2001; FAO, 2003). Aquaculture is significant on some Atlantic coasts particularly for local communities in Ireland, Spain and France. The regional aquaculture production in the Mediterranean show a sharp increased by about 185% in a decade (39 575 tonnes in 1984 to 113 103 tonnes in 1994) (Anonymous, 1999).

Aquaculture is taking place in coastal areas that are already impacted by human activities (McVey *et al.*, 2002). Higher nutrient levels in coastal locations are not inherently harmful as they provide the enrichment necessary for high productivity as in areas of upwelling (McVey *et al.*, 2002). A problem arises when the nutrient load is higher than the capacity of the natural processes to assimilate those nutrients. In other words, the system is out of balanced (McVey *et al.*, 2002).

In China, where annual aquaculture production is 4.5 million tonnes (wet weight) of red and brown marine macroalgae, seaweeds are significant nutrient removers. Production of *Laminaria japonica* (Japanese kelp) alone removes approximately 25 110 tons of nitrogen each year, considering a 2.79% dw N content and a wet to dry ratio of 5:1 (Egan and Yarish, 1990). Total biomass production by *Porphyra* is lower but its N content can go above 7% dw (Chopin and Yarish, 1998; Chopin *et al.*, 1999). Fei (2004) estimated that, on average, 4.4kg of N will be removed from the water with every ton wet weight of some of the common cultivated species like *Laminaria*, *Porphyra* and *Gracilaria*.

Due to its high surface/volume ratio, *Porphyra* spp. are fast growing, capable of rapid assimilation of N and phosphorus (P). This fact, together with its economic value,

makes the genus one of the most promising for bioremediation purposes and integrated aquaculture (Kraemer and Yarish, 1999; Neori *et al.*, 2004). Preliminary studies by Chopin *et al.* (1999) showed 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.

It is generally accepted that successful agriculture depends on vascular plant ecology and physiology for a basic understanding of crops. In the same way, successful mariculture depends on an 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 and Harrison, 1994; Craigie and Shacklock, 1995). The scientific basis for designing an efficient, profitable cultivation system has come from research into species and strains, the sites, and the environmental factors affecting productivity (Troell *et al.*, 2003; Neori *et al.*, 2004). As Chopin *et al.* (1999) pointed out, the efficacy of the different species of *Porphyra* as nutrient scrubbers needs to be compared in order to select the best suited for bioremediation. Recently, Carmona *et al.* (submitted) and Kraemer et al. (submitted; Kraemer *et al.*, 2004.) compared the nutrient uptake by native Northeast U.S. species with Asian species in short- and long-term experiments.

Porphyra dioica, a native species of the eastern North Atlantic, also appears to be a good candidate for aquaculture applications (Pereira *et al.*, submitted). 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.*, submitted), a form of vegetative propagation never described for this genus, but ideal for a periodically harvested crop.

In integrated aquaculture conditions, N will be in the form of ammonium and nitrate. Nitrate will result from bacterial nitrification, usually by a bacterial biofilter,

while ammonium will be derived from excretion by the animal component of the integrated system. Seaweed species may differ in their preferable N forms for assimilation (D'Elia and DeBoer, 1978; Hanisak, 1983). A species capable of growing equally well with those two N forms will have advantages in an integrated aquaculture environment.

Another important aspect is the daily cycle of nutrient uptake. Seaweeds, as photosynthetic organisms, likely perform differently under light or dark periods. In order to better estimate the overall efficiency as nutrients removers it is necessary to know their performance during the dark periods. Differences in NH_4^+ and NO_3^- during a diurnal cycle were shown for phytoplankton (Glibert and Garside, 1992). Cohen and Neori (1991) also showed that ammonia uptake rate of *Ulva lactuca* decreased during the night at fluxes of approximately 25 μmol l^{-1} h^{-1}. Diel rhythm of phosphate uptake rates for three species of microalgae, in P-limited conditions, was also reported by Ahn *et al.* (2002). These authors showed that for *Ankistrodesmus convolutus* and *Chlorella vulgaris* the P_i uptake rates increased during the daytime and decreased at night. In contrast, *Chlamydomonas* sp. exhibited the opposite uptake patern.

Nitrogen primarily, but at times also phosphorus, may limit algal growth in temperate marine regions (Howarth, 1988; Krom *et al.* 1991). These are also the main nutrients released by fish aquaculture, in a N:P proportion of ca. 7:1 (Chopin *et al.* 1999). Considering that N will be available far in excess in fish-aquaculture effluents, it is necessary to verify if any benefits would result from P enrichment.

This work provides information about the nutrient uptake efficiency of *Porphyra dioica*. The main objective is to determine how well this species can cope with levels of nitrogen much higher than in nature, but found in aquaculture effluents. Which form of nitrogen is better assimilated, ammonium or nitrate. This work will also determine the

influence of the diurnal cycle on the nutrient uptake and the effects of the simultaneous presence of nitrate and ammonium in the medium. Another objective will be to determine the effects of P enrichment and the consequent unusually low N:P ratios on the growth of the gametophytes of this species.

MATERIAL AND METHODS

Young gametophytes of *Porphyra dioica* 1 to 3 cm long and approximately 4 weeks old were used for these experiments, except when stated otherwise. Isolates were obtained from a conchocelis culture originated from tissue collected in Porto, Portugal (41°19'37"N, 8º45'40"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 in our previous work (Pereira *et al.*, in press).

Conditions common to all treatments were temperature (15ºC), photon flux density (PFD, 150 μmol photons m^{-2} s^{-1}), and photoperiod (12:$\overline{12}$ h, L:$\overline{D}$ cycle). Light was provided by cool-white fluorescent tubes. Gametophytes of *P. dioica* were grown in 1 litre flasks with Von Stosch's enriched seawater (Ott, 1965) modified to provide the desired nitrate, ammonium and phosphate concentrations. The algae were always acclimated for one week to the experimental temperature, PFD and photoperiod conditions.

Ammonium vs. Nitrate Uptake

Dissolved inorganic nitrogen (DIN) was added as either $NaNO_3$ or NH_4Cl in order to obtain concentrations of 25, 75, 150 and 300 μM of N as nitrate (NO_3^-) or ammonium (NH_4^+), respectively. For the NO_3^- enrichment there was also a 500 μM treatment. Phosphate (PO_4^{3-}) was added as $Na_2HPO_4.12H_2O$. The final N:P in the medium varied between 4 and 8 to 1. Three replicates per each N source and concentration were used. The stocking density of the cultures was 0.3 ± 0.05 g fw l^{-1} and the medium was replaced twice each week. Growth was measured by the change in biomass fresh weight (fw) in each flask. At each time the stocking density was reduced to the initial value. Periodically, samples of tissue were collected for pigments and carbon, hydrogen and nitrogen analysis (CHN). Every time the medium was changed 100 ml of the medium was filtered and frozen for water analysis. The experiment lasted three weeks and all tissues were acclimated one week under the common experimental temperature, PFD and photoperiod regimes.

Diel Nitrogen Uptake Studies

For this experiment algae were grown in 1 litre flasks with 0.8 litre of modified Von Stosch's enriched seaweed water (Ott, 1965). Dissolved inorganic nitrogen (DIN) was added as $NaNO_3$ and NH_4Cl in order to obtain concentrations of 300 μM NO_3^- and 50 μM NH_4^+, respectively. Phosphate (PO_4^{3-}) was added as $Na_2HPO_4.12H_2O$. The nutrient concentration was 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 during the dark period were performed inside the walk-in-chamber, under 5 to 7 μmol photons m^{-2} s^{-1} of red light. At the end of each growing period, water samples were collected, filtered and frozen for later water analysis.

Phosphorus Enrichment Studies

Young gametophytes of *Porphyra dioica* used for these experiments were 2 to 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 source of N, was maintained at the same concentration. Phosphate concentration varied to obtain N:P ratios of 3:1, 2:1, 1:1 and 1:2. The N and P concentrations were 171 (±5.0) μM of NO_3^- and 60 (±4.9), 84 (±8.5), 140 (±5.0) and 400 (±39.2) μM of PO_4^{3-}. Growth was measured by the change in biomass fw in each of the three replicate flasks. The medium was renewed twice a week. Every time the medium was changed, 100 ml of the medium was filtered and frozen for water analysis. The experiment lasted 4 weeks.

Water analysis

Samples from the incubated mediums were analyzed for inorganic N and P by the Environmental Research Institute, University of Connecticut, using a Four Channel Auto Analyzer equipped with High-Sensitivity Seawater Cartridges (Lachat - QuikChem AE Ion Analyzer, Hach Company, Loveland, Colorado). The Environmental Research Institute is an U.S. Environmental Protection Agency certified laboratory.

CHN Analysis

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

Pigment Analysis

The material collected during the experiments was stored at -20ºC. The analysis of phycobiliprotein (PBP) concentrations was performed in aqueous crude extracts, following the method described by Beer and Eshel (1985) with some modifications. Samples of 0.03 to 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 in cold, in dim light, and the extracts were kept in the dark at 4ºC overnight. The extracts were centrifuged at 10,000G for 20 min and the supernatants were used for phycobilin 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). Concentrations of phycoerythrin (PE) and phycocyanin (PC) were calculated using the formulas described by Beer and Eshel (1985).

For chlorophyll *a* (Chl *a*) analysis, we followed a procedure adapted from Lobban and Chapman (1988). Briefly, the pellet from the phycoerythrin extraction was collected, a few milligrams of $MgCO_3$ were added and the material was ground in 4 ml, 90% acetone. After centrifuging for 20 min. at 10,000G, the supernatant was collected

and the pellet used to repeat the extraction procedure. The two extraction volumes were combined and the absorbance at 665 nm was measured using a Perkin Elmer spectrophotometer (UV/VIS spectrophotometer Lambda 20, Perkin Elmer Analytical Division of EG & G, Wellesley, MA, USA).

Statistical Analysis

For all the treatments, three independent replicates were analyzed, and means and standard deviations were calculated. For each species, differences among treatments were tested for significance using two-way ANOVA. Multiple post-hoc comparisons among means were tested by the Student Newman Keuls (SNK) test. In all cases, the null hypothesis was rejected at the 5% significance level, according to Sokal and Rohlf (1995).

RESULTS

Ammonium vs Nitrate Uptake Studies

The specific growth rate of the gametophytes of *Porphyra dioica* was significantly influenced ($P<0.01$) by the nitrogen concentration in the medium for both N sources. Growth rate reached a maximum of 25% fw day^{-1}, at 300 μM of nitrogen (Fig. 1) and did not increase significantly ($P>0.05$) from 300 to 500 μM of nitrate. The

nitrogen source (ammonium vs. nitrate) did not affect growth rate (P>0.05). The Michaelis-Menten model fits well the curves of both N sources. The r^2 was 0.9901 and 0.9695 for nitrate and ammonium, respectively.

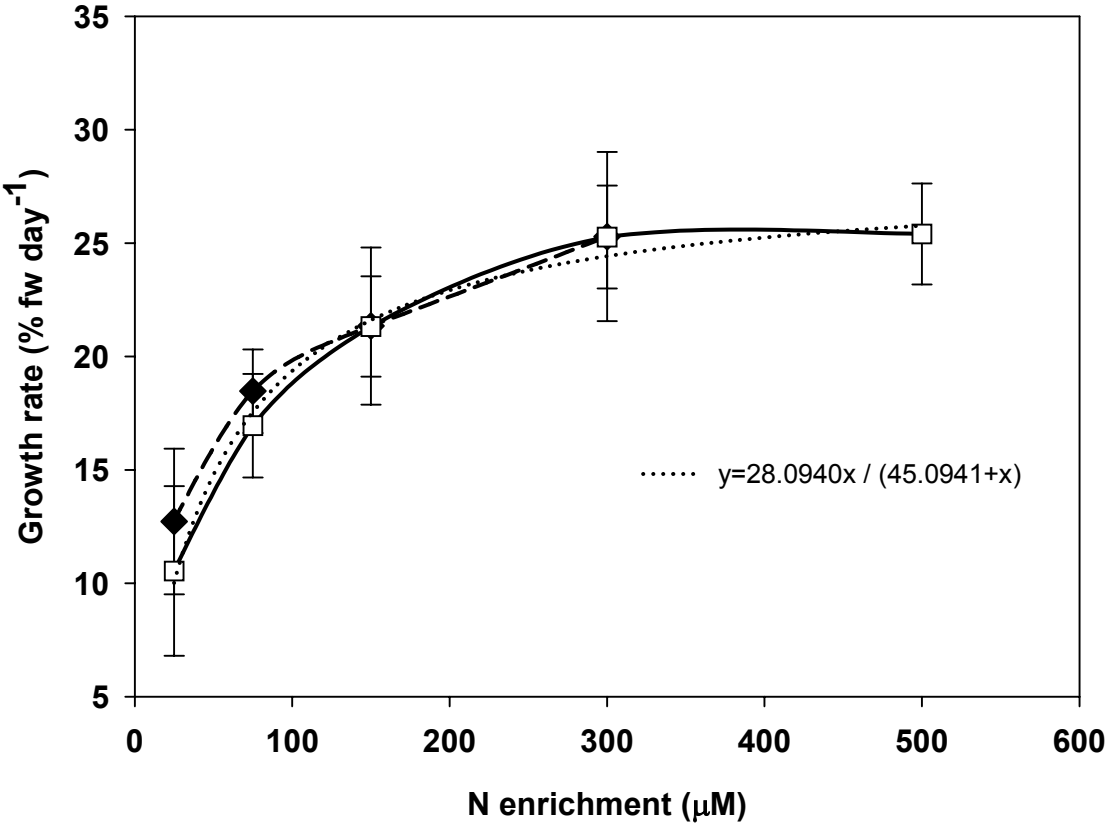

Figure 1: Relationship between growth rate of *Porphyra dioica* and nitrogen enrichment with two nitrogen forms, ammonium (◆) and nitrate (□). The dotted line (….) shows the Michaelis-Menten model fitted to the nitrate data. Other conditions, 15ºC, 12:$\overline{12}$ h, L:$\overline{D}$ cycle and 150 μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Dots are the means of three replicates per condition.

The N content of the plants increased significantly (P<0.05) and linearly with the increase in the N concentration (Fig, 2). The r^2 was 0.9756 for the NO_3^- enrichment experiment and 0.9900 for the NH_4^+ enrichment experiment. The form of N did not affect tissue N level (P>0.05). The highest N content was 4.9% dry weight (dw), recorded at the highest concentration tested, 500 μM of nitrate. If growth rate of the

plants was plotted against their N content (Fig. 3), the data once again fitted the Michaelis-Menten model. The r^2 for the nitrate enrichment experiment and for the ammonium enrichment experiment was 0.9126 and 0.9293, respectively. Thalli with approximately 4% N in the tissue reached a maximum growth rate of 25% fw increase day^{-1}. This was true for both nitrogen forms.

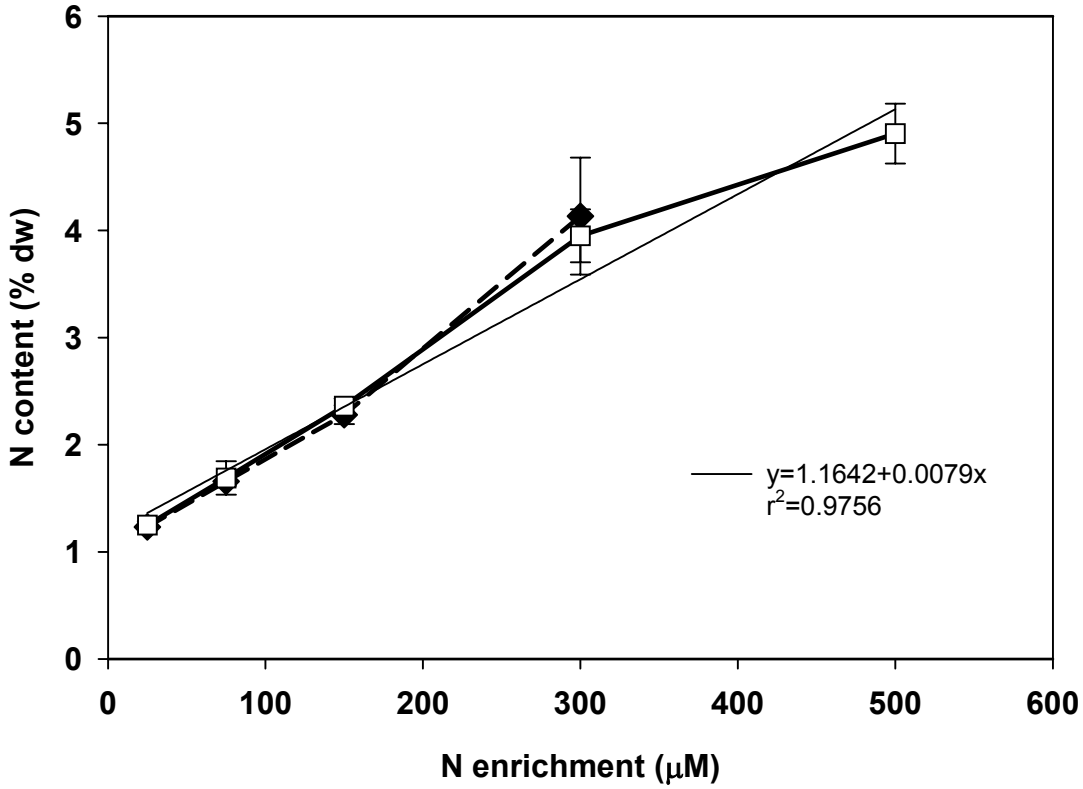

Figure 2: Nitrogen content of gametophytes of *Porphyra dioica* under different nitrogen concentrations and two nitrogen forms, ammonium (◆) and nitrate (□). Other conditions, 15ºC, 12:$\overline{12}$h, L:$\overline{D}$ cycle and 150 μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Dots are the means of three replicates per condition.

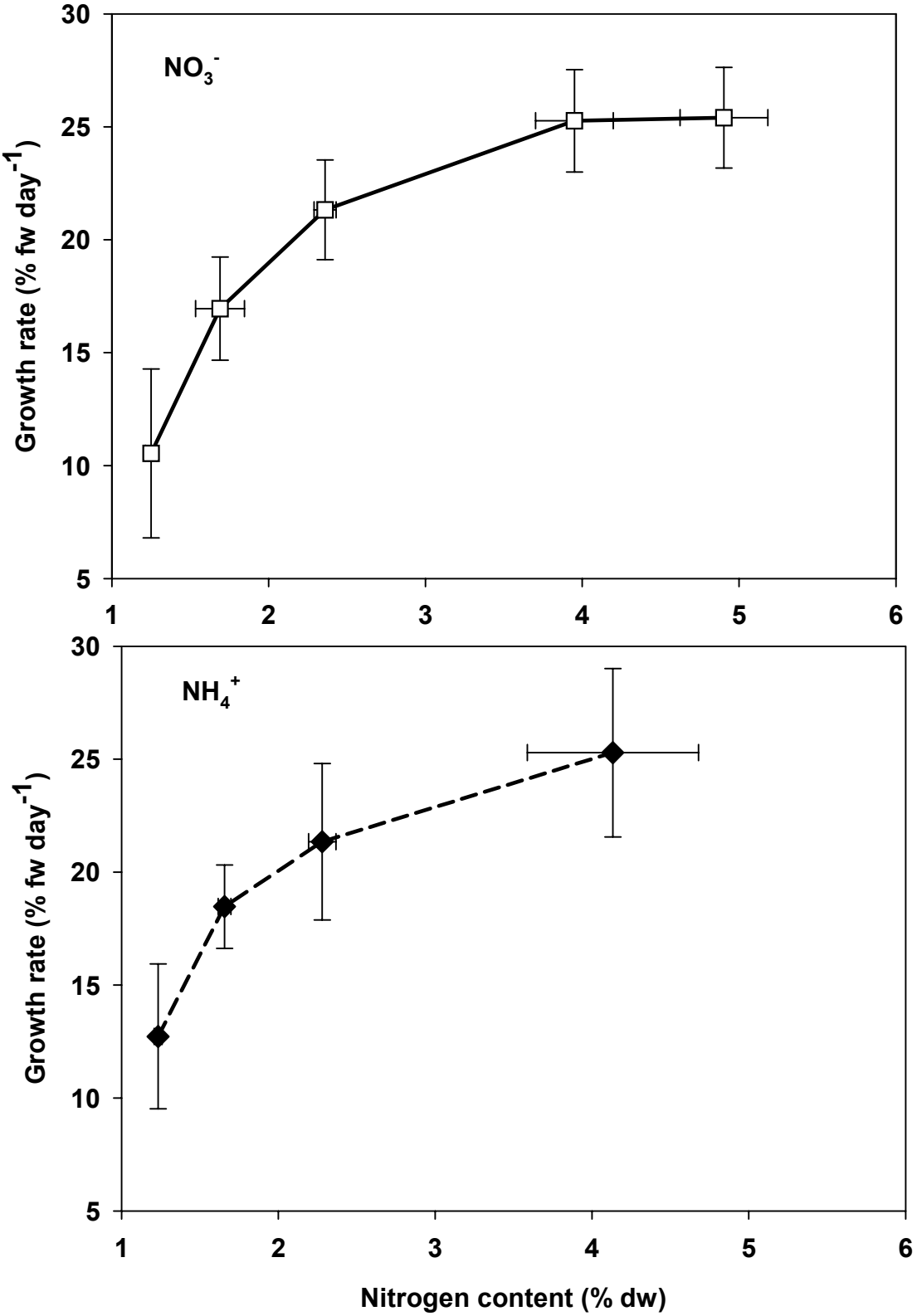

Figure 3: Relationship between growth rate and nitrogen content of gametophytes of *Porphyra dioica*, under different nitrogen concentration and two nitrogen forms, ammonium (◆) and nitrate (□).Other conditions, 15ºC, 12:$\overline{12}$, L:$\overline{D}$ and 150 µmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} of stocking density. Three replicates per condition.

The carbon content of the tissue did not vary significantly with the different nitrogen concentrations or forms. The values recorded were between 37 and 39.5%. On the other hand, the C:N ratio decreased significantly ($P<0.05$) with the increase in the nitrogen concentration in the medium. The decrease of the tissue C:N ratio was similar for both nitrogen forms, 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.

In terms of nutrient removal (Fig. 4), the amount of N removed from the medium increased almost linearly with the increase in the N concentration, for both nitrogen forms.

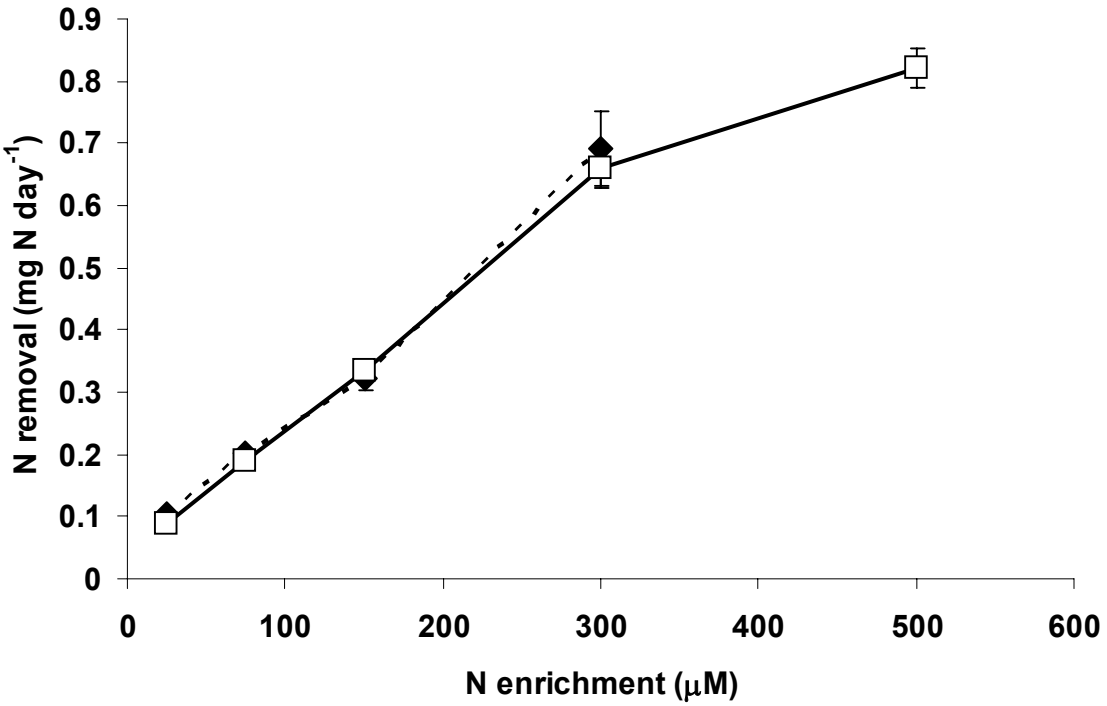

Figure 4: Nitrogen removal by gametophytes of *Porphyra dioica* under different nitrogen concentrations and two nitrogen forms, ammonium (◆) and nitrate (□). Other conditions, 15ºC, 12:$\overline{12}$h, L:$\overline{D}$ cycle and 150 μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Dots are the means of three replicates per condition.

The ANOVA indicated non-significant differences between the two N forms ($P>0.05$). In the nitrate enrichment experiment, where we had an additional condition with 500 μM of nitrogen, the removal did not increase as much as, for instance, from 150 to 300 μM. With a 100% 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 nitrate (Fig. 2).

At all concentrations used, the inorganic N in the medium was totally removed by the seaweeds after 4 days (Fig. 5). This was true for both nitrogen forms.

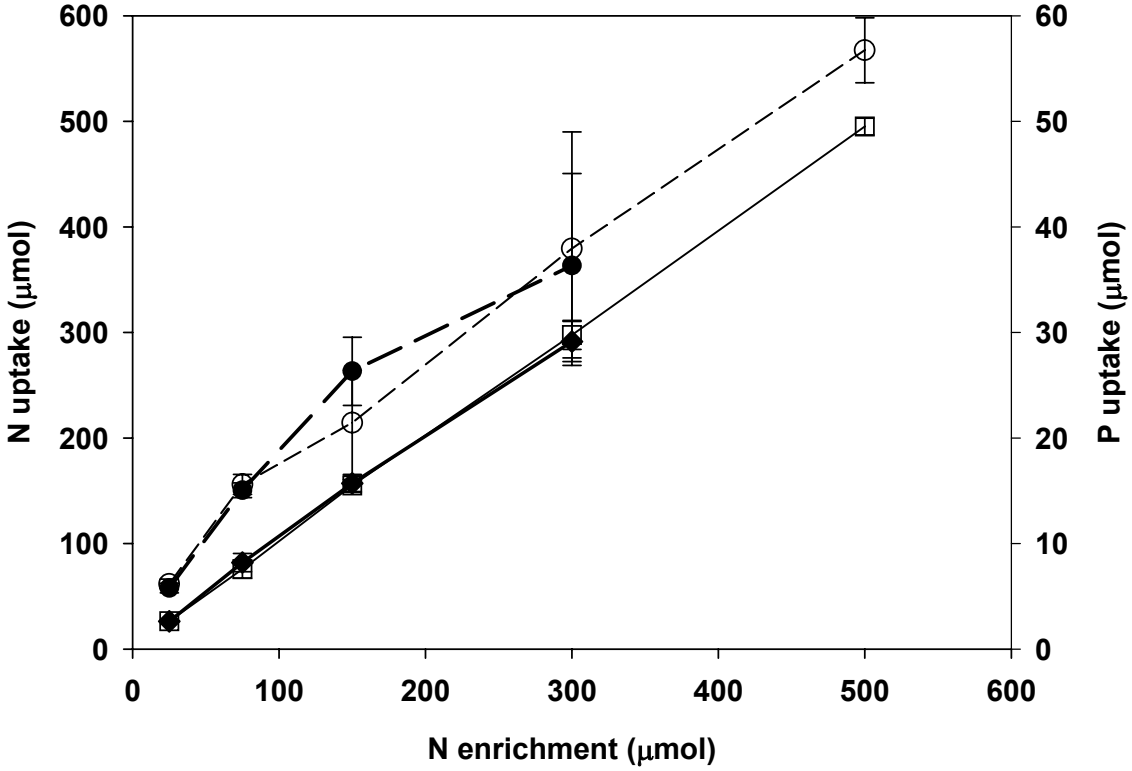

Figure 5: Amount of nitrogen and phosphorus removed from the medium by gametophytes of *Porphyra dioica*, after 4 days under different nitrogen concentrations and two nitrogen forms. Ammonium uptake (◆), nitrate uptake (□), phosphate uptake in medium with ammonium (●) and phosphate uptake in medium with nitrate (○). Other conditions, 15ºC, 12:$\overline{12}$h, L:$\overline{D}$ cycle and 150μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. 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. Three replicates per condition.

The percentage of nitrogen that was incorporated into new tissue (Fig. 6) varied significantly between different N concentrations of the medium. At 25 μM, all N removed from the medium was incorporated into new tissue. At higher concentrations only a fraction of the nitrogen removed was incorporated into new tissue. *Porphyra* with 75 μM of nitrogen available were able to incorporate into new tissue approximately 75% of the N removed. *Porphyra* in the treatments with 150 and 300 μM incorporated 63 and 65%, respectively. The thalli grown with 500 μM of nitrate incorporated 47% of this amount. These differences apply to both nitrogen forms, as seen in Fig. 6.

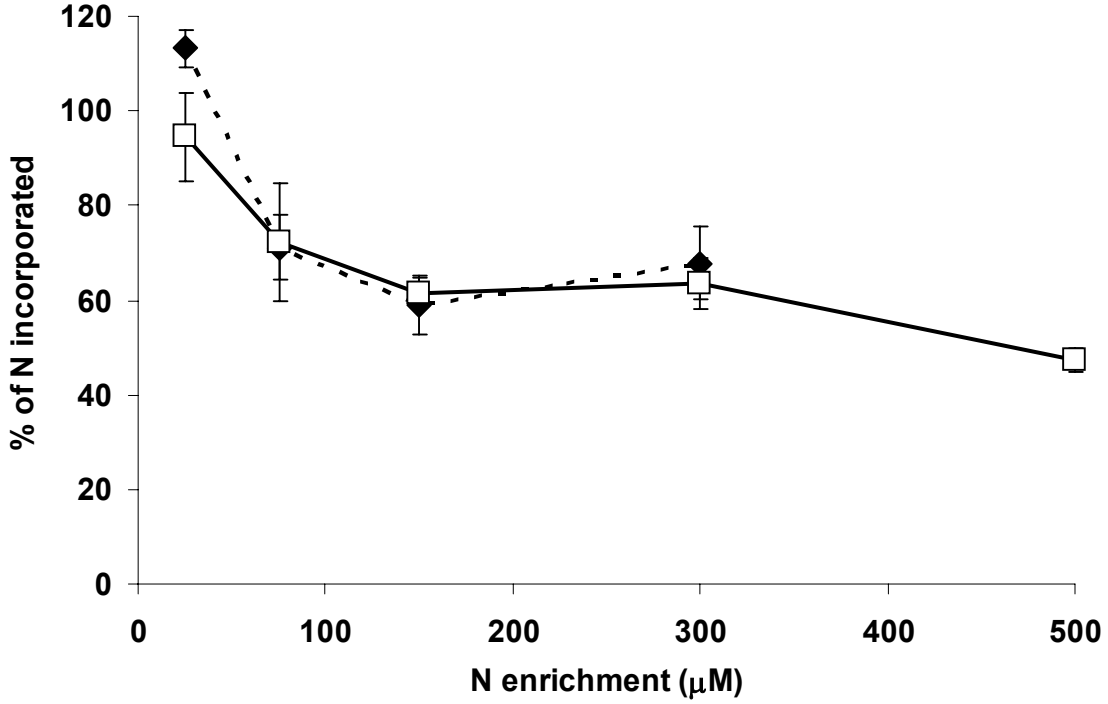

Figure 6 : Mean percentage of the nitrogen removed from the medium that was incorporated into new tissue by gametophytes of *Porphyra dioica* under different nitrogen concentrations and two nitrogen forms, ammonium (◆) and nitrate (□). Other conditions, 15ºC, 12:$\overline{12}$ h, L:$\overline{D}$ cycle and 150μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Three replicates per condition.

The uptake of PO_4^{3-} varied with the concentration available. The amount of PO_4^{3-} removed from the medium increased linearly with the increasing availability in the medium (Fig. 5). The percent of available P that was removed from the medium decreased progressively from 88 to 70% when NO_3^- concentration varied from 25 to 300 μM. When NH_4^+ was used, the PO_4^{3-} uptake percentage 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% for NH_4^+ concentrations of 25, 75, 150 and 300 μM, respectively.

The pigments content, PBP and Chl *a*, increased significantly ($P<0.05$) with the increase of the available nitrogen in the medium. PBP concentration was not influenced by the two N forms ($P>0.05$; Fig. 7). 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 forms, was the increase from 25 to 75 μM of N, which caused no significant effect on the PBP content. The maximum PBP content, 3.2 mg g^{-1} fw, was recorded at 300 μM NO_3^-. The phycoerythrin (PE) increased, in average, from 0.18 to 1.75 mg g^{-1} fw, at 25 and 300 μM, respectively. When the nitrate concentration was increased from 300-500 μM, the PE content of the thalli increased 62%. With 500 μM of nitrate the PE content of the tissue reached 2.84 mg g^{-1} fw.

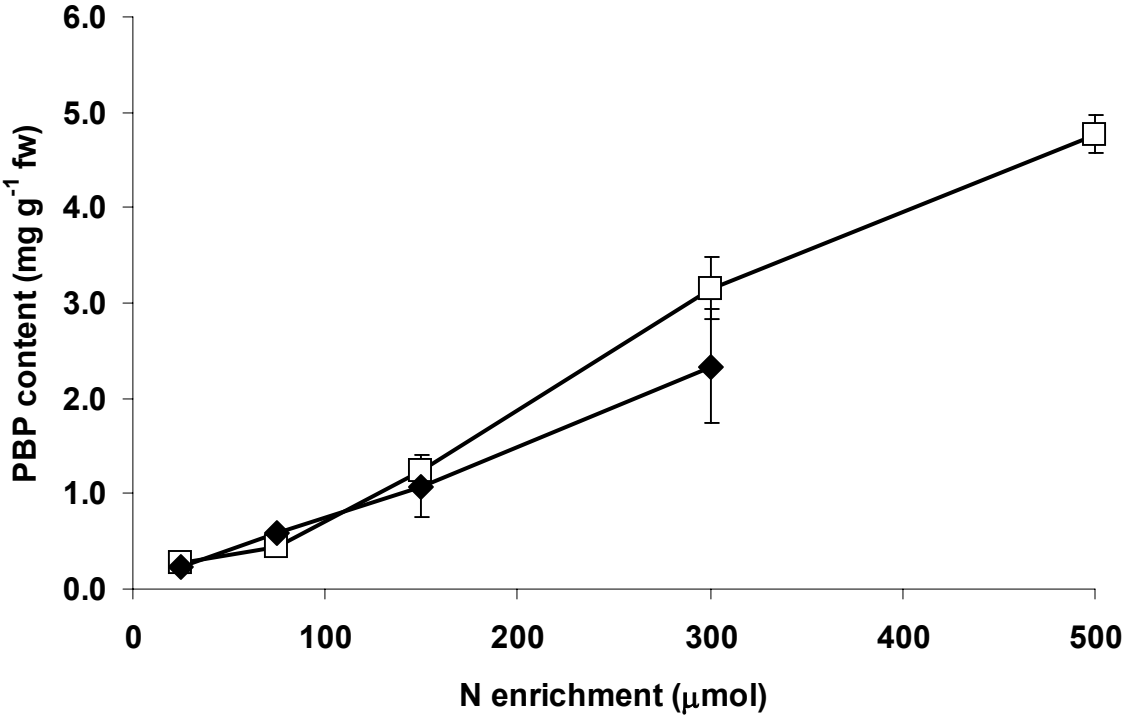

Figure 7: Phycobiliprotein content of thalli of *Porphyra dioica* under different nitrogen concentrations and two nitrogen forms, ammonium (◆) and nitrate (□). Other conditions, 15ºC, 12:$\overline{12}$h, L:$\overline{D}$ cycle and 150 μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Dots represent the mean of three replicates per condition.

Phycocyanin (PC) content at 300 μM was significantly higher in the thalli with NO_3^- than in those with NH_4^+ as source of N ($P<0.05$). *Porphyra* using NO_3^- had an average PC content of 1.17 mg g^{-1} fw, against only 0.82 mg g^{-1} fw in the thalli using NH_4^+. The Chl *a* content of the tissue was significantly different between the two nitrogen forms, being always higher in the thalli that used NH_4^+ as source of N (Figure 8).

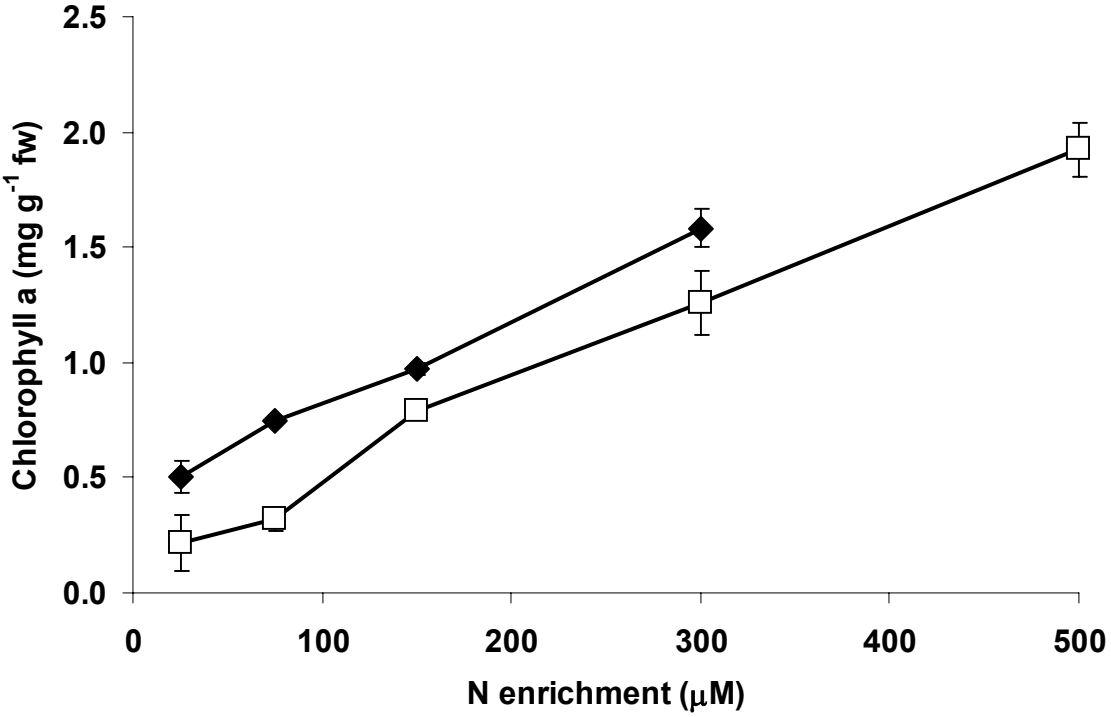

Figure 8: Chlorophyll *a* content of thalli of *Porphyra dioica* under different nitrogen concentrations and two nitrogen forms, ammonium (◆) and nitrate (□). Other conditions, 15ºC, 12:$\overline{12}$, L:$\overline{D}$ and 150 μmol photons m^{-2} s^{-1}, 0.3 g fw l^{-1} stocking density. Dots represent the mean of three replicates per condition.

Diel N Uptake Studies

Only the uptake of ammonium showed a clear diurnal influence (Figure 9). The algae were able to remove from the medium 70% (±9.6) of the available ammonium during the light period, while only 35% (±5.2) of the ammonium was removed during the dark period. These differences in ammonium uptake percentage are statistically significant ($P<0.001$).

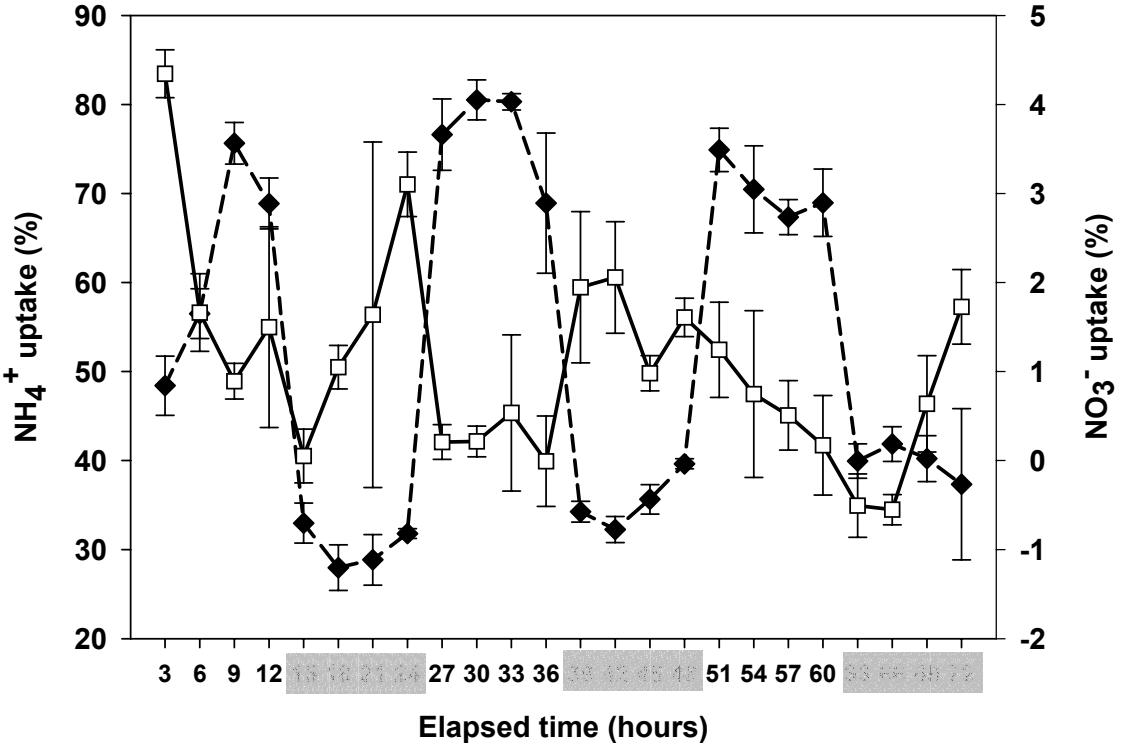

Figure 9: Mean diel variations (n=3) in uptake percentage of ammonium (◆) and nitrate (□) by gametophytes of *Porphyra dioica*. Medium consisted of VSE with 50 µM NH_4^+, 300 µM NO_3^- and 35 µM of PO_4^{3-}. Other conditions controlled included, 15ºC, 12:$\overline{12}$ h, L:$\overline{D}$ cycle and 150 µmol photons m^{-2} s^{-1}. Shadowed areas in the x axis represent the night periods.

The percentage of nitrate removed from the medium was always much lower than the percentage of ammonium. 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). The average NO_3^- removal for the three days was 1.1% during night period and only 0.7% during the light period. Although Fig. 9 seems to reveal a tendency for higher nitrate uptake during the night than during the day period, the data are not statistically different ($P>0.05$). The between-replicate variation in uptake percentage was higher for nitrate

than for ammonium. The phosphate uptake was also not influenced by the day and night cycle, and remained relatively stable throughout the experiment (Fig. 10).

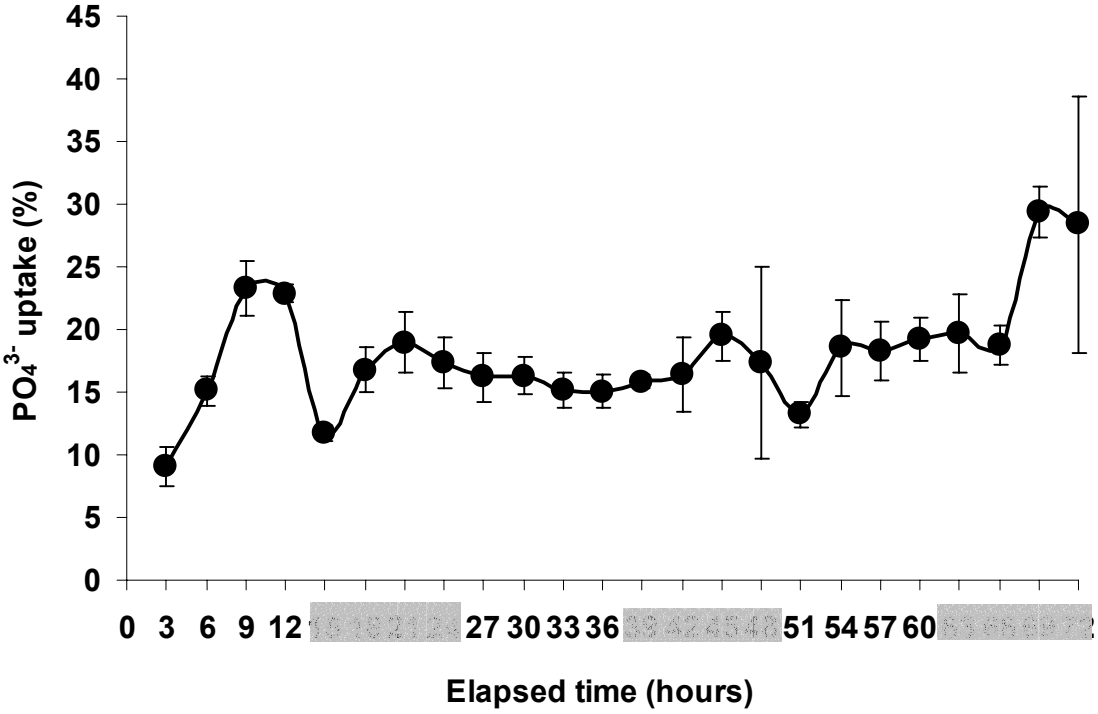

Figure 10: Mean diel variation (n=3) in uptake percentage of phosphate by gametophytes of *Porphyra dioica.* Medium consisted of VSE with 50 µM NH_4^+, 300 µM NO_3^- and 35 µM of PO_4^{3-}. Other conditions controlled included, 15ºC, 12:$\overline{12}$h, L:$\overline{D}$ cycle and 150 µmol photons m^{-2} s^{-1}. Shadowed areas in the x axis represent the night periods.

Phosphorus Enrichment Studies

During each culture period (3 - 4 days), *Porphyra dioica* removed almost 100% of the NO_3^- in the medium. The percentage of PO_4^{3-} removed decreased with the increase in PO_4^{3-} concentration, correspondent to the decrease in the N:P ratio (Fig. 11). This variation in the percentage of PO_4^{3-} uptake corresponds, however, to a constant

amount removed, from 47 to 51 µmol PO_4^{3-}. Unlike the NO_3^- uptake, the uptake of PO_4^{3-} fluctuated depending on the culture period, 3 or 4 days, before medium replenishment.

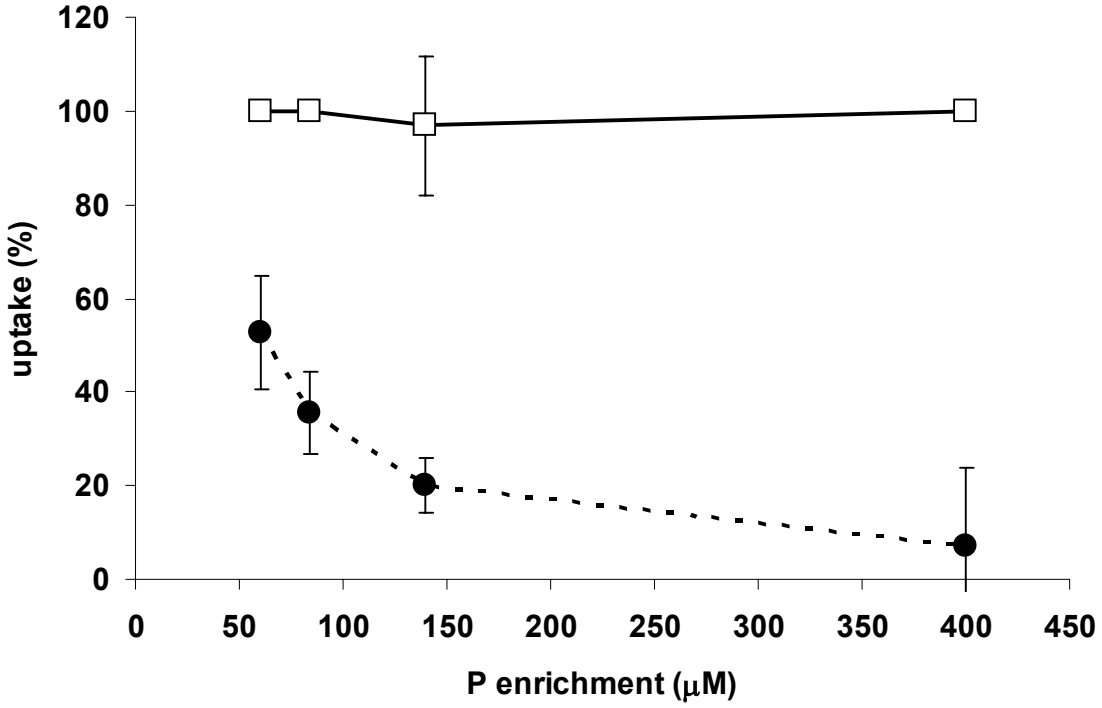

Figure 11: Mean uptake percentage of NO_3^- (□) and PO_4^{3-} (●) by gametophytes of *Porphyra dioica* in different P concentrations. Other conditions controlled, 15ºC, 12:$\overline{12}$ h, L:$\overline{D}$ cycle, 150 µmol photons m^{-2} s^{-1} and 171(±5) µM NO_3^-. Three replicates per condition.

More PO_4^{3-} was removed at the end of each 4 day period (61% in average) that at the end of each 3 day period (44% in average, for the 3:1 N:P ratio) (Fig. 12). This was more evident at the highest N:P tested, but was true for all N:P ratios. Growth rate of the gametophytes did not change significantly within the range of N:P ratios tested in this experiment, varying from 18.1 to 18.7% fw increase day^{-1}.

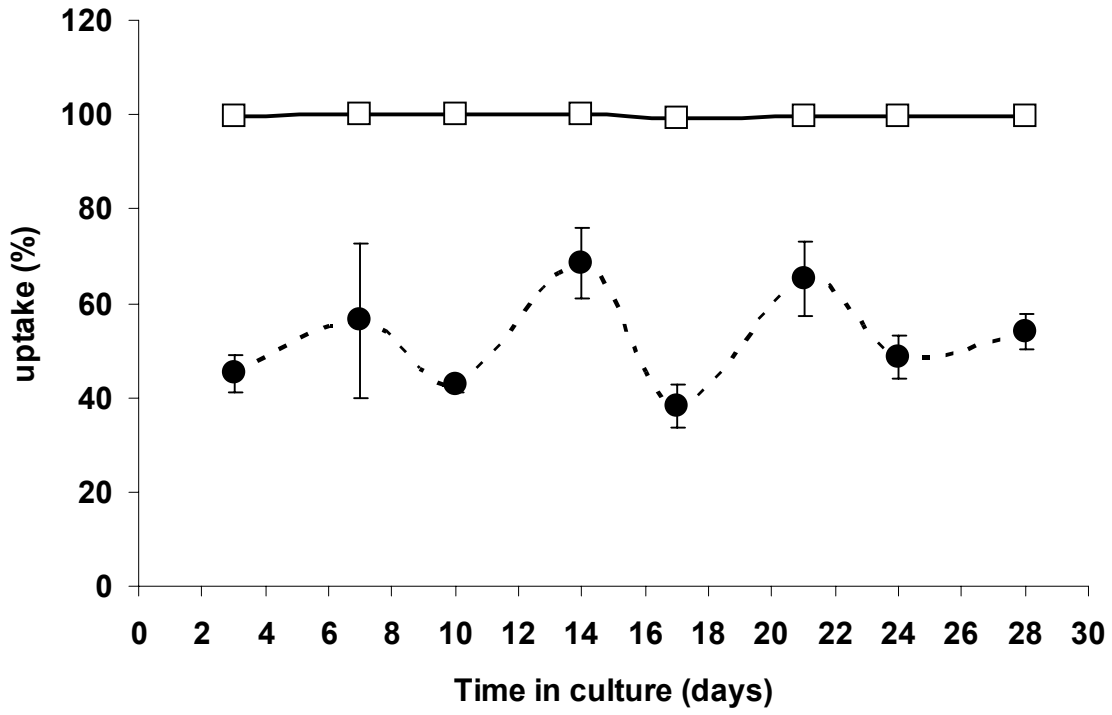

Figure 12: Mean uptake percentage of NO_3^- (□) and PO_4^{3-} (●) by gametophytes of *Porphyra dioica* after each period of 3 to 4 days before medium renovation. Medium consisted of VSE with 178 µM NO_3^- and 58 µM of PO_4^{3-}, i.e., N:P = 3. Other conditions controlled, 15ºC, 12:$\overline{12}$ h, L:$\overline{D}$ cycle and 150 µmol photons m^{-2} s^{-1}. Three replicates per condition.

DISCUSSION

The increase in growth rate observed with increasing N availability was expected. *Porphyra* has the characteristics of an opportunistic species, fast growth and capacity to deal with high nutrient concentrations. The growth rates obtained are in accordance to those obtained for this species in previous experiments, whenever N is not limiting (Pereira *et al.*, submitted). Carmona *et al.* (submitted) recorded maximum growth rate of 25% for *Porphyra amplissima* and 18% for *P. yezoensis* and *P.*

umbilicalis in conditions similar to the ones in our experiment. Nonetheless, it is interesting to note that *P. dioica* is able to grow equally well using NO_3^- or NH_4^+ as source of N. Not all macroalgae can grow equally well on these two sources. Studies with *Porphyra yezoensis* (Wu *et al.*, 1984; Amano & Noda, 1987) showed better growth and uptake rates when NH_4^+ was the source of N in comparison to NO_3^-. On the other hand, Hafting (1999) found that under high light (160 µmol photons m^{-2} s^{-1}) *P. yezoensis* grew better when NO_3^- was the N source. Carmona *et al.* (submitted), using *P. amplissima*, *P. leucosticta*, *P. purpurea*, *P. umbilicalis*, *P. haitanensis*, *P. katadai* and *P. yezoensis*, obtained results similar the ones we report in this study for *P. dioica*. 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) showed that *Gracilaria tikvahiae* grows better on NH_4^+ than on NO_3^- in laboratory conditions, whereas Lapointe and Ryther (1978) found that the same species grows equally well on these two N forms under high light outdoor tanks. In conclusion, some macroalgae grow better with nitrate, others grow better with ammonium and others grow equally well with both forms, but sometimes there are contradictory results. However, like Hanisak (1983) noted, some discrepancies in nutrient utilization studies may be due to toxicity caused by high levels of the added nitrogen.

For some seaweed, like *Ulva lactuca* (Waite & Mitchell, 1972), *Hypnea musciformis* and *Macrocystis pyrifera* (Haines & Wheeler 1978), concentrations of NH_4^+ over 30-50 µM may be toxic. That is not the case for *Porphyra dioica* at least up to 300 µM. Amano and Noda (1987) observed that, for *P. yezoensis*, ammonium is toxic only above 30 ppm ($\simeq$2100 µM). In the case of *P. umbilicalis*, levels of 1.4 mM NH_4^+ caused a decrease in growth rate, although not siginifcantly different than growth at 300 µM NH_4^+ (Carmona *et al.* submitted).

These data are relevant in terms of application of these species in integrated aquaculture. As mentioned earlier, NH_4^+ will be the primary form of N resulting from fish metabolism. Ammonium concentrations have to be controlled as they can be toxic for fish. Person-LeRuyet *et al.* (1995) reported 40 mg l^{-1} of total ammonia-N ($NH_3 + NH_4^+$) as a lethal concentration for 50% of the population of juvenile seabass, *Dicentrachus labrax*. Nonetheless, NH_4^+ concentrations between 60-100 µM can be recorded in fish effluents (Neori *et al.*, 1996). Hussenot (2003) measured an annual average of 150 µM and 84 µM of dissolved inorganic nitrogen (DIN) in the effluents of two intensive sea bass and turbot grow-out systems. Although most of the ammonium will be transformed into nitrate, seaweed species able to cope with high levels of NH_4^+ become advantageous for integrated aquaculture, reducing the amount of bacterial bioconversion necessary.

The N content of the thalli increased significantly with the increases in the N concentration in the medium in a manner similar to that for growth rate. However, unlike growth rate, N content increased linearly and does not seem to reach a maximum at least up to 500 µM of nitrate and 300 µM of ammonium. This resulted in a proportional increase in the N removal without reaching a clear asymptote. In fact, the algae were able to remove all N available in all concentrations and both N forms.

The observed increase in N content did not always correspond to an increase in growth rate. For both N forms, a tissue N content over 4% dw did not result in significantly higher growth rates. This agrees with our observation in previous experiments (Pereira *et al.*, submitted), where plants growing under similar N concentration and PFD had a growth rate of 26.5% fw day^{-1} with a N content of 5.4% dw. Although the study was not designed for that purpose, a critical N concentration (*sensu* Hanisak, 1983) of ca. 4% dw can be suggested for *Porphyra*

dioica. This is the estimated N content above which the tissue N content will have no effects on the growth rate of *P. dioica*. The critical tissue N level is 2% dw for *Gracilaria tikvahiae* (Hanisak, 1990) and 1.9% dw for *Codium fragile* (Hanisak, 1979). Fujita *et al.* (1989) recorded, for *Ulva rigida*, critical N levels of ≤ 2.4% dw and 3.0% dw depending on the N source, NO_3^- and NH_4^+, respectively. According to Pedersen and Borum (1996) *Ulva lactuca* has critical tissue N of 4% dw. Other *Porphyra* species showed critical N levels similar to the one suggested by our results with *P. dioica*. 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) determined also for *P. yezoensis* a critical N content of 0.4% fw (4.0% dw considering a fresh to dry ration of 10), regardless of the N source (NH_4^+ or NO_3^-).

The percentage of N incorporated into new tissue decreased with the increase in N concentration in the medium. We have seen from previous experiments (Pereira *et al.*, submitted) that *Porphyra* does not incorporate all the nitrogen that they can remove from the water into tissue. The photosynthetic pigments, Chl *a* and PBP responded positively to the increase 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 micosporine 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, as reported by Peinado (2003) for *P. umbilicalis* and *P. columbina*. Gröniger *et al.* (1999) reported a MAA content of 10-15 mg g^{-1} dw in *P. umbilicalis*. On the other hand, FAA contents reported are > 4.75 g/100g dw for *Porphyra* spp. (Harada *et al.*, 1990) and ca. 4.0 g/100 g dw for *P. yezoensis* (Niwa *et al.*, 2003). In both cases the four major amino acids (alanine, aspartic acid, glutamic acid and taurine) amounted to about 90% of the total FAA.

Free amino acid composition is affected by nutrient source and concentration (Bird *et al.*, 1982; Horrocks *et al.*, 1995). Pedersen *et al.* (submitted), showed that *Porphyra purpurea* responded positively and significantly to increased nutrient availability, increasing its tissue N, FAA and pigment content. Nisizawa and Oofusa (1990) noted that the increase in the amount of total pigments is parallel to the organoleptic value. On the other hand, the taste of *Porphyra* is known to be correlated with its amino acid content, namely alanine, glutamic and aspartic acids (Nisizawa & Oofusa, 1990) and also inosinic and guanylic acids (Noda *et al.*, 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 % of N incorporated, if we consider the possibility of release of organic molecules.

Tyler *et al*. (1994) reported for the first time the release of dissolved organic nitrogen (DON) by *Ulva lactuca* during active growth. The release of DON was also measured in studies with phytoplankton (Collos *et al.*, 1992; Bronk & Ward, 1999). On the other hand, Naldi and Wheeler (2002), using ^{15}N isotopes, observed very little release of DON by *Ulva fenestrata* and *Gracilaria pacifica*. The concentration of FAA in the medium increased significantly only on the first day and when NH_4^+ was the source of N. These authors suggested that the release of DON is more likely to be due to the degradation of detritus than from live algal tissue. In our study no detritus was apparent in the system.

Other possible explanation to the discrepancy between N removed and N incorporated into new tissue is that, although the N is removed from the medium, the plants did not have enough time to incorporate that much N into organic molecules.

However, if that is the case, then we could also expect some differences between the two N sources. Ammonium is known to be more rapidly used by the seaweed, since it can be directly incorporated into organic molecules, whereas nitrate has first to be reduced to ammonium (Lobban & Harrison, 1994).

Yet another hypothesis to explain the difference between N removal and N incorporation is the ammonia volatilization. The equilibrium between gaseous unionized ammonia (NH_3) and aqueous, ionized ammonium (NH_4^+) is strongly affected by pH and much less strongly affected by temperature (Hargreaves, 1998). As a crude approximation, this author refers that, at a pH 9.3, about 50% of ammonia is NH_3, and at a pH 8.3 about 10% is NH_3. We know from previous studies (Pereira et al., submitted) that *Porphyra dioica* elevates the pH up to 8.8. Therefore, it is possible that the higher growth rate at higher N concentrations was causing a faster increase in pH with consequent volatilization of some of the ammonia, which was not detected in the water analysis and was assumed as removed. This hypothesis does not, however, explain the same discrepancy for the nitrate-enriched media. Although our culture media was not axenic, all materials used were sterilized and denitrification by bacteria is unlikely to be significant.

Results of previous studies with different stocking densities of *Porphyra dioica* (Pereira *et al.*, submitted) are similar in that the fraction of N incorporated into new tissue was higher in the higher stocking densities, *i.e.*, when less nitrogen per thallus was available. Further studies are needed to determine the fate of the missing nitrogen. Dissolved organic nitrogen in the medium should be quantified as possible release products, but also as possible sources of N to support growth. Iwasaki (1967) found that several amino acids can be used as N sources by the conchocelis of *Porphyra tenera*.

Carbon : nitrogen ratios (C:N) 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" (Neish *et al.*, 1977). A similar inverse relationship between carbohydrates and N availability was also reported (Gerard, 1982; Rosenberg & Ramus, 1982). Gerard (1982) noted the increase in carbohydrate content in *Macrocystis pyrifera* in a low N environment. Rosenberg and Ramus (1982) reported also an increase in carbohydrates, in *Gracilaria foliifera* and *Ulva* sp. when growth may have been N limited. In the case of *Porphyra 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 increased, in average, nearly ten times in thalli grown with 300 μM NO_3^- compared with those with 25 μM NO_3^-. The maximum values of PE obtained in the study, 2.85 (±0.13) mg g^{-1} fw compare well with other *Porphyra* species. Conitz *et al.* (2001) reported 1.80 to 3.67 mg g^{-1} dw in young gametophytes of *Porphyra linearis* grown at 90-110 μmol photons m^{-2} s^{-1}, 8:$\overline{16}$h, L:$\overline{D}$ cycle although these authors used only 88 μM NO_3^-. Carmona *et al.* (submitted) reported 0.67 (±0.10) mg PE g^{-1} fw for *Porphyra amplissima*, 3.25 (±0.35) for *Porphyra purpurea*, 0.73 (±0.52) for *Porphyra haitanensis* and 0.70 (±0.08) mg PE g^{-1} fw for *Porphyra katadai*. In that study, these four species presented higher PE contents than those observed here, when NH_4^+ was used instead of NO_3^-. That tendency was not clearly observed for *P. dioica* in our study. Amano and Noda (1987) found that NH_4-N enriched media was more effective for the recovery of nutrient stressed and discoloured fronds of *P. yezoensis* than NO_3-N enriched media, *i.e.* increase in Chl *a*, PE and PC content.

The increase in PC with the increasing nitrogen concentration was greater when thalli were supplied with nitrate than with ammonium. From 25 to 300 μM, the PC

content of the thalli grown with NO_3^- increased 15 times, while that of the thalli with NH_4^+ increased 10 times. This observation is also contrary to the findings by Amano and Noda (1987). Just like for the PE content, those authors found that the recovery of PC content in discoloured fronds of *P. yezoensis* was higher when fertilized with NH_4-N compounds than with NO_3-N compounds.

These changes in the PBP content were expected as these pigments constitute important N-storage compounds in red algae (Bird *et al.* 1982). Phycoerythrin comprises 20% of total N of *Gracilaria tikvahiae* (Lapointe & Duke, 1984). In *Gracilaria 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 these of *Gracilaria* species. Based on a nitrogen-to-protein conversion factor of 4.92 (*cf.* 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.* (submitted) for *P. purpurea* (N content 6% dw, PE content 3.44 mg g^{-1} fw and assuming the same dry-to-wt weight ratio) we have that PE corresponds to 0.26% of the total N.

The results of the diel uptake experiment showed that *Porphyra dioica* prefers ammonium to nitrate when both forms are present. Although there was 6 times more NO_3^- available, the percentage of NH_4^+ removed was far superior. The algae received a daily dose of 2500 µmol of NO_3^- and 365 µmol of NH_4^+. During the light period, the average percentage removed equals to 17 µmol of NO_3^- and 255 µmol of NH_4^+ removed, respectively. During the night period the amounts removed corresponded to

29 and 128 μmol of NO_3^- and NH_4^+, respectively. In terms of PO_4^{3-}, the removal was similar during the day and corresponds to a removal of 14 μmol per day.

Nitrate uptake by phytoplankton is inhibited by ammonium concentrations as low as 1 μM (Conway, 1977). Some seaweed (especially kelp) are able to uptake NO_3^- and NH_4^+ simultaneously and at the same rate (Harrison *et al.*, 1986). Other seaweeds, in contrast, take up NH_4^+ preferentially over NO_3^- and, therefore, NH_4^+ inhibits the uptake of NO_3^- (Harrison & Hurd, 2001). Partial inhibition of nitrate uptake by ammonium is common in seaweeds (D'Elia & DeBoer, 1978; Haines & Wheeler, 1978; DeBoer, 1981). D'Elia and DeBoer (1978) reported that *Neoagardiella baileyi* and *Gracilaria foliifera* (Rhodophyta) preferred NH_4^+ over NO_3^- even when plants were preconditioned on NO_3^- as the sole N source. That is exactly the case in our study with *Porphyra dioica*. Those authors also report that NO_3^- uptake was suppressed at 5 μM NH_4^+ but simultaneous uptake occurred at lower concentrations. The uptake of NO_3^- by *P. dioica* is strongly affected but still occurs with ca. 50 μM NH_4^+. Thomas and Harrison (1985) showed that ammonium inhibited NO_3^- uptake by *Porphyra perforata* only for the first 10 to 20 minutes and then nitrate uptake rates were independent of ammonium concentration. They also found that this temporary inhibition does not occur if the blades were N starved for 8 days. These results are important in an integrated aquaculture system where ammonium and nitrate are the main sources of N in the fish-farm effluents, and will be available simultaneously. A species capable of assimilating simultaneously both forms of N have advantage over seaweeds in which NO_3^- uptake is inhibited in the presence of NH_4^+.

The NO_3^- uptake results during the N:P ratio experiment suggest that the algae were N limited. All NO_3^- was removed from the medium after each culture period, 3-4 days and the plants were growing 18% day^{-1}, below the maximum growth rate obtained

with higher NO_3^- concentrations. These results agree with the ones obtained under the same conditions during the NH_4^+ vs. NO_3^- uptake experiment. 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 reason of 5.75 ± 0.05. Hafting (1999) states that *Porphyra yezoensis* cannot store excess P over the range of loads tested (0.26 to 16.29 μmol day^{-1}). Species known to store P are *Ulva lactuca* and the brown alga *Pilayella* (Lundberg *et al.*, 1989). 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 1).

In the NH_4^+ vs. NO_3^- uptake experiment, the N:P uptake ratios increased from 4.3 to 8.7, with the increasing NO_3^- concentration from 25 to 500 μM. Since P was always in excess, it is reasonable to assume that it was taken up only according to the algae needs. Therefore, a N:P ratio of 7-9 seems to be enough for maximum growth *Porphyra dioica* when N is not limiting (Table 1), since those were the conditions yielding maximum growth rates. This N:P ratio is lower than 13-15, suggested by Hafting (1999) as the optimum for *P. yezoensis*, and much lower than the 30:1 average ratio suggested by Atkinson and Smith (1983) for seaweeds as a group. If considered isolated from other data, and in light of what is suggested by Harrison and Hurd (2001), the suggested N:P ratio would mean N limitation. However, as these authors also point out, each seaweed species may have a different optimum. Further studies should be designed in order to precisely determine the optimum ratio for *P. dioica*.

Table 1: Summary of N and P uptake performance

	Growth rate (% day^{-1})	Daily load (µmol)			Daily uptake (µmol)			Daily uptake (µmol)[a]		
Experiment		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	1.8	3.9	7	1.6	4.3	7	1.6	4.3
	17.0	21	4.3	5.0	21	3.8	5.6	21	3.8	5.6
	21.3	43	7.2	5.9	42	5.9	7.0	42	5.9	7.0
	25.3	86	16.1	5.3	84	11.3	7.4	84	11.3	7.4
	25.4	143	17.0	8.4	142	16.2	8.7	142	16.2	8.7
Diel-uptake	15.5	2912[b]	77.4	37.6	430	28.0	15.4	161	10.5	15.4

[a] Data normalized to 0.3g fw l^{-1} stocking density.
[b] In this experiment the N load is the sum of NH_4^+ and NO_3^-, same for uptake values.

The application of these laboratory results to the data available from the aquaculture industry will give an idea of the potential of *Porphyra dioica* for integrated aquaculture. The estimated amount of P and N released by ton of fish per year is 7.0 and 49.3 kg, respectively (Chopin *et al.*, 1999). 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 (141.5µmol-N and 16.2µmol-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. Besides, *P. dioica* has other advantageous characteristics. *Porphyra dioica* showed an equally good capacity to remove nitrogen as NO_3^- and as NH_4^+ from the culture medium. This is an important feature because, generally, intensive aquacultures have bacterial biofilters that transform NH_4^+ in 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^-. On the other hand, the preferential uptake of NH_4^+ over NO_3^- does not seem to inhibit completely the uptake of the NO_3^-, which also constitutes an important advantage. In conclusion, *Porphyra dioica* suggests to be an interesting species for integrated aquaculture and bioremediation. Further studies to investigate the potential of this species for direct human consumption and other uses are needed, to determine the economic value of its biomass. The use of algae with economic value is critical to assure the economic sustainability of the integrated aquaculture practices.

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CHAPTER 5

FINAL CONCLUSIONS

FINAL CONCLUSIONS

The studies of the life cycle of *Porphyra dioica* Brodie et Irvine showed that the species has a typical biphasic life cycle. The species can be included in the type IV life-cycle classification described by Notoya (1997), *i.e.*, the *Porphyra dentata* type. Reproduction through asexual spores was not observed in any phase of the life cycle of *P. dioica*. Despite the reported formation of young blades in the basal parts of older thalli, there was not any observation of release of asexual spores by the gametophytes.

The wide range of temperature tolerance (in culture) of the conchocelis phase possibly allows it to occur throughout the year. Gametophytes were found throughout the year. However, a continuous production of spores and constant renewal of the gametophytes population is unlikely to occur in nature, judging from our laboratory results. The release of conchospores, in the laboratory, required a combination of temperature, photoperiod and light intensity. Such combination of factors does not exist year round on the North Coast of Portugal. Our field work also showed that there is a clear increase of the population during winter and early spring months. With a constant renewal of the gametophyte population, we could expect its percent cover to be more stable throughout the year. However, other factors, such as herbivory or competition with other species, may prevent the establishment of the new individuals in parts of the year. Holmes and Brodie (2004) also report the presence of *Porphyra dioica* throughout the year in Sidmouth Bay (Devon, UK). They found young blades, < 1.0 g wet mass, during all year, which suggests a constant renewal of the population.

On the other hand, the fact that we were able to grow blades under all three photoperiods supports the idea of a possible continual renewal of the population. As mentioned in the introduction of this work, other factors besides temperature,

photoperiod and PFD can influence reproduction. Information on how long can the gametophytes of species like *Porphyra dioica* last in nature is non existent. From our experience, blades produced in the lab up to two-three months) and then reproduction is followed by disintegration. Males reproduce at SD, ND and LD photoperiods and 10-20ºC after 42 days in culture from the the time since conchospore release. Female-like thalli also stopped growing after 28 days of active growth, even without fertilization and loss of tissue by sporulation. Blades can be maintained for longer time periods in culture as long as they are kept under sub-optimal conditions for growth. Information about longevity of blades in nature can help solve the question of how this taxa maybe perennating along the Portuguese Coast. Dixon and Richardson (1969) describe their observations that the leafy thalli of *P. perforata* and *Bangia fuscopurpurea* perennate from basal fragments, from which vegetative cells are released or develop *in situ* to form new thalli. One important observation made during the present studies on the gametophytes of *Porphyra dioica*, was its ability to form young blades from the basal parts of adult thalli. This feature, never reported for *Porphyra* species, can explain the presence of *P. dioica* throughout the year along the North Coast or Portugal. It may have important implications for aquaculture of this species. The only report to a similar phenomenon is the work of Bird *et al.* (1972). These authors refer to the regeneration of small blades of *P. linearis* from thalli that necrosed in culture before sporulating.

Our results showed that the main factor controlling the growth of conchocelis is temperature. Conchosporangia production by *Porphyra dioica* requires a combination of short-days at 15ºC, without aeration, and the release of conchospores is promoted by a period of time under very low light (5-25 µmol photons m^{-2} s^{-1}). Aeration is also crucial for the germination of the conchospores and production of new blades.

One of the key factors for the success of *Porphyra* cultivation, as practiced today, is the establishment of a constant and readily available supply of "seedstock" of juvenile organisms (Yarish *et al.* 1998, 1999). This implies the existence of facilities and personnel to monitor and maintain the cultures of conchocelis under controlled conditions. The consequence for the industry is high production costs. The simplicity of requirements for the growth of conchocelis and production of conchospores by *P. dioica,* makes this a good candidate for cultivation. Species that require more specific conditions, and/or a specific chain of events to complete its life cycles, cause an increase of the costs of the operation. *Porphyra dioica* was able to achieve growth rates over 20% fw day^{-1} from 10 to 20ºC at 8:$\overline{16}$, 10:$\overline{14}$, 12:$\overline{12}$ and 16:$\bar{8}$ h L:$\overline{D}$ cycles. The effects of the temperature on the growth rate showed that *Porphyra dioica* is well adapted to the water temperatures in its natural habitat. The effects of photoperiod on the growth rate of the gametophytes were not surprising, since they are found in nature during all year. The gametophytes were able to grow in photoperiods with 8 to 16 hours of light per day. If this genus is acceptable for biofiltration of land-based finfish aquaculture, flexibility of the culture conditions will be important. Using eurythermal species provides an advantage for year round aquaculture and for biofiltration. The selection of a seaweed species for application in integrated aquaculture is frequently conditioned by the species of fish being grown and its temperature requirements.

The gametophytes of *Porphyra dioica* can sustain a high growth rate and productivity over a range of temperature, photoperiod and light conditions. The optimal conditions can be adapted to the objective of the culture. Stocking density of the culture is important in terms of biomass production. In combination with light, these factors influence the biochemical properties of the thalli. If the purpose of the culture is to obtain biomass with higher N content (> 6% dw), for instance for phycoerythrin

extraction, stocking densities of 0.3 and 0.1 g fw l^{-1} and 50 µmol photons m^{-2} s^{-1} should be used. If the focus is on biomass production and maximum nutrient assimilation, higher stocking densities and PFDs should be used. The use of PFD lower than 50 µmol photons m^{-2} s^{-1} to further increase the PE content needs to be investigated. At some point a low PFD is expected to influence negatively growth rate and productivity, and an eventual higher PE content might not compensate for this loss in biomass.

The capacity to regenerate new blades from the basal portions of adult blades constitutes and important advantage for *Porphyra dioica* to be used in aquaculture. It becomes even more interesting to note that such phenomena happened in temperatures from 10-20ºC and in different photoperiods. The growth rate of these new blades varied between 22 and 26% day^{-1}. It was similar to that of the "parental" blades and without differences among the photoperiods tested. The implications of these observations are easy to understand in light of what was previously said about the costs of *Porphyra* production. As discussed in the third chapter, much work has been done trying do develop ways of vegetative reproduction for *Porphyra* species. The objective is to develop new techniques for "seed" production, bypassing the need of conchocelis cultivation. The observation of this natural vegetative propagation characteristic in *Porphyra* would, at least theoretically, allow precisely that. The gametophytes could be cultured continuously, without the need of a periodic "seeding" procedure and the maintenance of conchocelis cultures. The possibility of a constant renewal of the gametophytes can allow several harvests without the need of the conchocelis phase.

Porphyra dioica showed equal capacity to uptake nitrogen as NO_3^- and as NH_4^+. This is important because, generally, intensive aquacultures have bacterial biofilters that transform NH_4^+ in NO_3^-. In that case, unless the seaweeds receive water directly from the fish tanks, most of the N available will be in the form of NO_3^-. On the other hand,

the results of the diel uptake experiment showed that *Porphyra dioica* prefers ammonium to nitrate when both forms are present. For this species, the uptake of NO_3^- is strongly affected but not completely inhibited by the presence of NH_4^+ and still occurs with ca. 45 μM NH_4^+. The implication of this for an integrated aquaculture is that *P. dioica* could be used before or after the bacterial biofilter, taking up more ammonium or more nitrate, respectively.

For application in bioremediation and integrated aquaculture, the daily load of nutrients is an important factor. Once determined that *Porphyra dioica* is able to cope with high nutrient concentrations, it is necessary to evaluate its performance in an environment with a continuous flux of nutrients. Nonetheless, the results obtained in this study indicate that *P. dioica* has good uptake efficiency of nitrogen and phosphorus. The main disadvantage of the use of *Porphyra* species as a biofilter in integrated aquaculture, has to do with its ephemeral life cycle. A good biofilter, besides being efficient removing the nutrients, should be stable and reliable. To use *Porphyra* species it would be necessary to replace the biomass every 1 to 2 months. After that period the blades can start releasing spores and the biomass can be lost very quickly. Hence the importance of further investigations on the vegetative propagation ability now reported for *P. dioica*.

In terms of nutrient uptake and assimilation, it is necessary to clarify the difference between the N removed from the water and the amount of N incorporated into new tissue. Short term uptake experiments, with several native *Porphyra* species from the Northeast of United States, showed no effects in the N content of the tissue after 20 minutes incubations, despite the removal of N from the medium (R. Carmona, personal communication).

Despite the natural constraints, it is important to try to extrapolate the results of small scale lab experiments into larger scales. For the potential N-removal results obtained in this study, and considering that approximately 600 g of N are released per day by ton of fish (data from a 50 fish pound, 36m^3 each, turbot and sea bass farm, as described by Costa *et al.*, submitted), 286 m^2 of *Porphyra dioica* culture would remove 80% of the N from the effluent. This is considering the seaweed cultivation in 1 m^3 seaweed tanks (1 m^2 surface area), with 1.5 g fw l^{-1} stocking density. In this conditions *P. dioica* is capable of removing 1.67 g N per day and per tank. For the same conditions, but using a stocking density of only 0.3 g fw l^{-1}, the area of culture needed increases to 300 m^2. Working on the same data, we must also keep in mind that the biomass of *Porphyra dioica* needed to achieve those results is a function of the growth rate and N content of the thalli (Fig. 1). An extrapolation based on the results obtained show that, in ideal conditions, maximizing growth rate and N content, the biomass necessary would be less than 100 kg. However, in the laboratory, this combination of high growth rate and high N content was achieved only in the lower stocking densities (0.1 g fw l^{-1}). Follow up studies should be done in a continuous flux system, in an integrated aquaculture system. Under those conditions it will be possible to test the influence of a continuous supply (higher daily doses) of nutrients and prevent possible CO_2 limitations. It should be then possible to achieve higher growth rates with higher stocking densities.

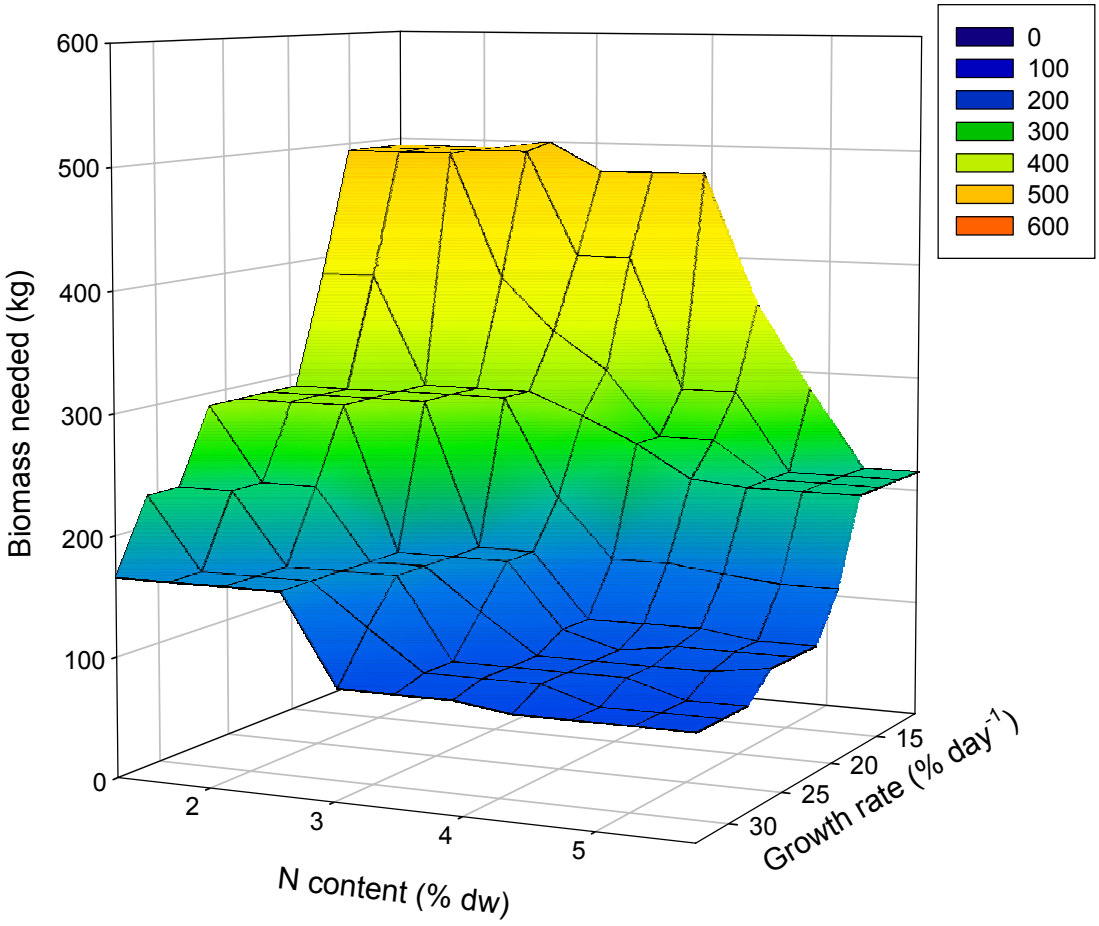

Figure 1 – 3D simulation model of the biomass of *Porphyra dioica* needed to achieve a 80% reduction in a N load (during 12 hours light day) of 600 g N day^{-1} (195 l min^{-1} x 150 µM N).

In conclusion, *Porphyra dioica* proved to be an interesting species for integrated aquaculture and bioremediation. Its growth rate is one of the highest reported for *Porphyra* species and its nutrient removal capacity compares well with other species already tested for that purpose. Another important advantage of this species is its ability to achieve growth rates over 20% day^{-1} over a range of temperatures and photoperiods. The possibility of vegetative propagation of the blades is a very interesting feature that deserves further investigation. If confirmed in scaled up studies, this feature will add

one more important advantage to the use of this species in aquaculture. Other important aspects of the physiology of *Porphyra*, that need further investigation, are those related with nutrient uptake and quality of the biomass. In terms of the nutrient uptake, more studies are needed on the effects of phosphorus and N:P ratio and also on the rates of incorporation of the nutrients into new tissue. The quality of the biomass has direct implications on the value of the species. The amino acids profile should be investigated, as well as the ways to increase the content of interesting amino-acids like taurine. *Porphyra* is also a good source of ω-3 fatty acids (Sánchez-Machado *et al.*, 2004). Other promising applications of *Porphyra* are the extraction of mycosporine like amino acids, useful for UV protection, and the use of *Porphyra* as food additive for fish or other animals. In summary, there are several possible valuable substances on *Porphyra*, besides the PE, which can be affected by the culture conditions. This alga will continue to be an interesting model for physiological studies as well as a promising organism for biotechnology research.

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