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Integrated aquaculture of Bonnemaisoniaceae: physiological and nutritional controls of biomass production and of halogenated metabolite content

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Área de especialização Botânica Marinha

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Tese orientada por Prof. Dr. Rui Orlando Pimenta Santos

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Dedico este trabalho à minha mãe

Sei que se sentiria orgulhosa…

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Resumo

O uso de algas como biofiltros de efluentes de cultivo de animais marinhos ainda não foi adoptado pela indústria da aquacultura. A investigação deverá focar os seus esforços na tentativa de domesticar algas com valor comercial, de modo a que a biofiltração de nutrientes seja vista pela indústria da aquacultura como uma tecnologia auto sustentável, amiga do ambiente e que produz grandes quantidades de uma biomassa que pode ser rentável. As espécies do género *Asparagopsis* possuem um elevado conteúdo e diversidade de compostos halogenados voláteis (CHVs) que são já explorados comercialmente pela indústria da cosmética. Nesse sentido, o cultivo integrado de *Asparagopsis* com animais marinhos deve ser considerado como uma oportunidade. O meu objectivo nesta tese foi o de tentar estabelecer o cultivo da fase tetrasporófita das espécies *A. armata* e *A. taxiformis* e comparar as respectivas capacidades para remover nutrientes e produzir biomassa com as de espécies de *Ulva*, até então as espécies mais sucedidas nestes sistemas de cultivo integrado. Com o objectivo de determinar as condições de cultivo que maximizam a remoção de nutrientes dos efluentes, a produção de biomassa e os níveis internos de CHVs, foram exploradas as respostas fisiológicas das espécies a diferentes níveis dos recursos que são controláveis em cultivo (luz, azoto e carbono).

A performance das espécies de *Asparagopsis* em cultivo integrado excedeu a performance das espécies de *Ulva*. Neste sistema de cultivo, 5 g de peso fresco L^{-1} foi considerada a densidade óptima de inoculo nos tanques e 3 volumes por hora, a taxa ideal de renovação de effleuntes nos tanques das algas. Estas condições garantem a quantidade de nutrientes, mas especialmente de CO_2 que maximizam a biofiltração de nutrientes, a produção de biomassa e o nível interno de bromofórmio (CVH maioritário nestas espécies). Em condições limitantes de CO_2 para a fotossíntese, ocorre um decréscimo significativo do crescimento e da produção de compostos secundários à base de carbono como os CHVs. Não foi possível conseguir o cultivo anual de ambas as espécies neste sistema de cultivo, porque a temperatura dentro dos tanques das algas ultrapassou os 27 e os 29 ºC, temperatures letais para *A. armata* e *A. taxiformis*, respectivamente.

Summary

The use of seaweed as biofilters of animal mariculture discharges has not been widely adopted by the aquaculture industry. Research efforts should focus on the cultivation of novel seaweed species with economic value so that nutrient biofiltration may be identified by the aquaculture industry as a self sustainable, environmental friendly technology that produces profitable biomass. The *Asparagopsis* spp. volatile halogenated compounds (VHCs) are explored for cosmetics formulations and so the integrated aquaculture of *Asparagopsis* species should be considered as an opportunity. In this thesis I aim to establish the tank domestication of the tetrasporophyte phase of *Asparagopsis* species (*A. armata* and *A. taxiformis*) and compare its nutrient biofiltration and biomass production performance with the most successful seaweed biofilters, *Ulva spp*. By exploiting the physiological responses of the species to different levels of the manageable resources in culture (light, nitrogen and carbon) I aim to determine the cultivation conditions that maximize the TAN removal from the effluents, the biomass production of the system and the internal levels of VHCs.

The performances of *Asparagopsis* species in integrated aquaculture exceeded that of the *Ulva* spp. In this integrated cultivation system, the optimal *Asparagopsis* spp. stocking density (light) was 5 g fresh weight L^{-1} and the ideal supply rates of fish effluents to the seaweed tanks was ~3 vol h^{-1}. These conditions provided the the quantity of nutrients, but especially CO_2 to maximize the nutrients biofiltration, the biomass yield and the bromoform (the major VHC in these species) internal levels. At CO_2 limiting conditions for photosynthesis non–structural carbohydrate pools are affected, decreasing both growth and the production of carbon based secondary compounds. The continuously year round cultivation of both *Asparagopsis* species in this system was not possible, because the tank water temperature surpassed 27 and 29 ºC, lethal for the cultivation of *A. armata* and *A. taxiformis*, respectively.

CHAPTER 1

General Introduction

Integrated fish/seaweed aquaculture: rationale

Aquaculture, the fastest growing food-producing sector, presently accounts for almost 50 percent of the world's food fish and is perceived as having the greatest potential to meet the growing demand for aquatic food (FAO 2006). Rapid development of in-land intensive fed aquaculture (e.g. fish and shrimp) has raised increasing concerns on the environmental impacts of such mono-specifics practices in the adjacent coastal waters (Folke and Kaustky 1992, Costa-Pierce, 1996). In general, some 85% of phosphorus, 80–88% of carbon and 52–95% of nitrogen input into a marine fish culture system as feed may be lost into the environment through feed wastage, fish excretion, faeces production and respiration, which in turn may induce eutrophication, harmful algal blooms and anoxia (Wu, 1995). The economic success of these intensive mono-aquaculture practices has in part to do with the fact that farmers do not have to internalize the cost of water treatment. However, with all the awareness raised by scientists, industry, politicians and especially consumers that such technologies are no longer considered sustainable, farmers will soon have to pay for the remediation of the environmental impacts caused by their operations (Chamberlaine and Rosenthal 1995, Costa-Pierce 1996, Naylor et al. 2000, Chopin et al. 2001).

Several sustainable approaches have been proposed to coastal aquaculture (Folke and Kautsky 1989, Wurts 2000, Frankic and Hershner 2003, Neori et al. 2004). To mitigate the impacts of dissolved inorganic nutrients, two main practical approaches have been used so far: bacterial and plant (including algae) biofilters. Bacterial biofilters are dissimilative, which means that the biofilters transform the most toxic nutrients into harmless forms through a series of oxidation and reduction processes but then released them into the environment (van Rijn 1996). Biofiltration by plants is considered extractive. Using solar energy and the nutrients wastes from the mariculture practices, plants photosynthesize new biomass and reduce the overall release of nutrients to the environment. The wastes of one resource consumer become a resource (fertilizer) for the production of other organism, the so called integrated multi trophic aquaculture (IMTA). Such a balanced ecosystem approach provides nutrient bioremediation capacity, mutual benefits to co-cultured organisms, and economic diversification by producing other value-added profitable products (Chopin et al. 2001).

Research on seaweed biofilters for the treatment of dissolved inorganic nitrogen and phosphorus derived from effluents of land based mariculture practices started in the mid-1970s (Ryther et al. 1975, Langton et al. 1977) and continued in the 80s with few isolated works (e.g., Chang and Wang 1985, DeBusk et al. 1986, McDonald 1987). In the 1990s, this research field gained a renewed and increased interest especially undertaken in Israel (Vandermeulen et al. 1990, Cohen and Neori 1991, Shpigel et al. 1993, Neori et al. 2000), Spain (Canary Islands – Jiménez del Rio et al. 1994, 1996) and Chile (Buschmann et al. 1994). So far, seaweed biofiltration of fish farm effluents has not been widely adopted by the aquaculture industry, probably because the technology in itself is costly. Its future development may probably depend on the progress of new regulations that enforce the fish farm companies to internalize the environmental costs of their operations (polluter-paying

principle). In the meanwhile, research efforts should focus on the cultivation of economic valuable seaweeds so that nutrient biofiltration may be identified by the aquaculture industry as a self sustainable, environmental friendly technology that produces profitable biomass using a free source of nutrients and CO_2.

Tested seaweed species

Several seaweed species have been tested for their biofiltration potential either cultivated on a laboratory scale or in outdoors small pilot scale. However, only *Ulva* and *Gracilaria* species were tested and successfully cultivated in bigger scale inland integrated outdoor cultivation systems (see review by Neori et al. 2004).

Species from the genus *Ulva* (Chlorophyta) were soon identified as ideal candidates for filtering fish effluents, due to their capacity to quickly absorb and metabolize nitrogen, their high growth rates, low epiphytism susceptibility, controlled life cycle and their world wide distribution (Jiménez del Rio et al. 1996, Neori et al. 2000, Msuya and Neori 2002, Mata and Santos 2003, Schuenhoff et al. 2003). The drawback of using these species is their limited after-market and relatively low-value. In some of these integrated cultivation studies the produced biomass was used as feed for an algivore component of the integrated system (Neori et al. 1998, 2000).

The genus *Gracilaria* (Rhodophyta) has a commercial value in established markets, such as agar-agar, human consumption and as fodder for high-value marine herbivores. The use of *Gracilaria* spp. as biofilters has been adopted in regions where its culture in ropes or in ponds was already traditional. Farmers are now moving their cultivation units to zones near effluents of shrimp or fish ponds or cages to exploit the nutrients excess as a resource

input (Buschmann et al. 1994, Troell et al. 1997, Fei et al. 2000, Nelson et al. 2001, Zhou et al. 2006). Inland cultures of *Gracilaria* species in tanks or ponds allowed in some cases the development of epiphytes or other contaminating algal species within the cultures (Friedlander et al. 1991, 2001, Friedlander 1992, Haglund and Pedersén 1993). This implies a proper management of the cultures, which may limit the success of these species as biofilters.

Specimens from the economically important red seaweed genus *Porphyra* were studied as biofilters in the USA and Canada (Yarish et al. 1999, 2001). They showed interesting characteristics in terms of biomass production and biofiltration rates and plus have a high and established market value. However, the genus life cycle is complex, which makes it very difficult to maintain year-round growth of a purely vegetative culture.

It urges to find novel seaweed species that can work as profitable biofilters. Species with a potential high market value should be selected and tested in these integrated cultivation system. Ideally, they should be able to combine all the favourable characteristics of the *Ulva* spp. in these systems. Under cultivation, it may be possible to exploit the physiological characteristics of the species to maximize their biofiltration and biomass production rates. Red algae species are particularly interesting, because their natural products have demonstrated activity against a wide range of organisms, from bacteria to fish (McConnel and Fenical 1979, Hay et al. 1987b, de Nys et al. 1995, Wright et al. 2004, Nylund et al. 2005).

Why *Asparagopsis* species?

Chemical features

Species of the rhodophyte family Bonnemaisoniaceae are known to produce a wide variety of halogenated metabolites (Fenical 1975, McConnell and Fenical 1977a, b, 1980, Rose et al. 1977). The genus *Asparagopsis* Montagne alone is a particularly prolific source releasing over 100 of such volatile halogenated compounds (VHCs) mainly brominated, such as bromoform ($CHBr_3$), but also smaller amounts of other bromine, chlorine and iodinated methanes, ethanes, ethanols, acetaldehydes, acetones, 2-acetoxypropanes, propenes, epoxypropanes, acroleins, butenones and several halogenated acetic and acrylic acids (Burreson et al. 1976, Woolard et al. 1976, 1979, McConell and Fenical 1977a). Besides the diversity of compounds, the members of this family have the particularity of concentrating these compounds in specialized structures known as gland cells (Wolk 1968, Fenical 1975, Marshall 2003, Paul et al. 2006a, b). Wolk (1968) estimated that the tetrasporophyte phase of *Bonnemaisonia hamifera* concentrates both bromine and iodine in these gland cells respectively 30- and 3-fold higher than in neighbouring cells.

With this combination of diversity and quantity of secondary compounds, the Bonnemaisoniaceae species extracts act remarkably in antifouling assays (de Nys et al. 1995). When screened together with other seaweed taxa, they (Bonnemaisoniaceae species) usually show the strongest and broadest spectrum of antimicrobial activity (Hornsey and Hide 1974, Pesando and Caram 1984, Reichelt and Borowitzka 1984, Ballesteros et al. 1992, Bansemir et al. 2006, Salvador et al. 2007). These characteristics attracted the attention of a cosmetic company, which patented a special technique to extract the compounds to act as natural preservatives in cosmetics formulations, as anti –dandruff and scalp cleanser and as anti–acne treatment (Algues et Mer 2002). *Asparagopsis* species have also been shown to

produce sulphated galactans with promising therapeutic applications (Braun et al. 1983, Caporiccio et al. 1983) and new sources of anti-HIV compounds (Haslin et al. 2001).

Considering the potential high-value market of the *Asparagopsis* genus secondary metabolites, its species should be considered as serious candidates for mass cultivation integrated with fish farm units.

Morphological and biological characteristics

Two *Asparagopsis* species are currently recognized: *A. armata* Harvey and *A. taxiformis* (Delile) Trevisan (Dixon 1964, Dixon and Irvine 1977, Bonin and Hawkes 1987). Thalli of *Asparagopsis* are composed of sparsely branched, creeping stolons and erect shots from which numerous side branches develop in all directions with a plumose appearance (Børgesen 1915). *Asparagopsis* constitutes the gametophytic (haploid) life stage in a diplohaplontic heteromorphic life cycle (Feldmann and Feldmann 1939, 1942, Chihara 1961, 1962). The epiphytic tetrasporophytic '*Falkenbergia'* stage is composed entirely of densely ramified filaments consisting of three cell rows. Feldmann and Feldmann (1942) identified the tetrasporophytes of *A. armata* and *A. taxiformis* as *F. rufolanosa* (Harvey) Schmitz and *F. hillenbrandii* (Bornet) Falkenberg, respectively.

Asparagopsis armata seems to be a temperate species. It is native to southern Australia and New Zealand (Horridge 1951) and is now found from the British Isles, passing through Portugal, the Canary and Salvage Islands to Senegal as well (Dixon and Irvine 1977, Price et al. 1986). *Asparagopsis taxiformis* has a typical tropical to warm temperate distribution; it abounds throughout the tropical and warm-temperate parts of the Atlantic and Indo-Pacific (Abbott and Williamson 1974, Price et al. 1986, Bonin and Hawkes 1987). During this thesis, the presence of *A. taxiformis* was detected in the southern Portuguese coast (Berecibar

pers. comm.) and its provenience genetically confirmed to be from the Mediterranean lineage (Andreakis et al. 2007).

Physiological considerations to optimize mass algal cultures

Basic research on the physiology of the seaweed species is necessary to develop and improve the process of their domestication in tanks. It is thus important to have a proper understanding of the impacts of environmental variables on the biomass production, and inherently on the nutrient removal rates. Optimal rates in tank land-based culture of seaweeds may be achieved by adjusting ambient parameters such as light, temperature, salinity, nutrient availability and carbon (pH) supply. Laboratory-based photosynthetic and growth experiments are important to understand fundamental physiological processes. However, these experiments conducted under highly controlled conditions may only allow us to predict responses or understand the results obtained in commercial production, where many factors vary simultaneously and on a long-term basis. It is important as well to follow how those ambient parameters affect the biomass production in the outdoor cultivation system.

Light

Light is the source of energy for photosynthesis in plants, thus affecting growth. The light available to the seaweeds in aerated tanks is very particular; individuals are alternately exposed to the surface (bright sunlight) and plunged to the depths of the tank (virtual darkness). The study of the relationship between several light levels and photosynthesis (P/I

curves) is a fundamental aspect of the physiological basis for cultivation. The response curve patterns allow us to understand how the species photosynthetic performance reacts under the saturating and sub-saturating irradiances in the tanks. Another important aspect to consider is that at the surface high levels of photosynthetically active radiation (PAR) may be a threat to the plant metabolism if the irradiance exceeds the demands of photosynthesis (Osmond 1994, Aguirre-von-Wobeser et al. 2000). It is important to know whether the plants are capable of photoacclimation or if they are photoinhibited at any time of the day. Ultimately, the optimum light regime for the seaweeds in aerated tank cultivation is usually found by regulating the biomass density inside the tanks.

Nitrogen biofiltration / nutrition

An adequate nitrogen nutrition is critical to maximize the performance of the species in culture because of its role in amino acid, nucleic acid and protein biosynthesis and ultimately in growth. The nitrogen uptake and biomass growth kinetics is usually interpreted in terms of the Michaelis-Menten model, increasing with its availability in the medium until saturation (D'Elia and DeBoer 1978, Haines and Wheeler 1978). N deficiency (supply below the threshold) will clearly limits the growth and the N removal capacity of the system.

Total ammonia nitrogen (TAN) is directly excreted by fish and is the main dissolved inorganic nitrogen form present in the effluents of flow through fish cultivation systems. Most of the seaweed species prefer to take up TAN over other forms of nitrogen (D'Elia and DeBoer 1978). In on-shore tank seaweed cultivation systems it is possible to have total control of the nitrogen nutrition by adjusting the fish effluents turnover rates to the seaweed tanks. The TAN fluxes to the seaweeds can be adjusted, so as to maximise the biomass yield, the TAN removal capacity or the TAN removal efficiency (Neori et al. 2003).

Carbon nutrition (pH)

Adequate carbon nutrition is also an essential requirement for successful algal cultivation. In general, in dense biomass cultivation tanks the algae quickly depletes the dissolved inorganic carbon (DIC) pool in the water, suffering from carbon malnutrition and consequent low rates of production (Bidwell et al. 1985, McLachlan et al. 1986, Jiménez et. al. 1995). In traditional seaweed cultivation systems carbon nutrition can be controlled by pH-regulated additions of carbon. The variables to control are the chemical form (bicarbonate or carbon dioxide) in which the carbon is added to the cultures and the pH set point at which is added (Bidwell et al. 1985, Craigie and Shacklock 1989, Amat and Braud 1993, Demetropoulos and Langdon 2004). However, the use of extra carbon sources represents a major operational cost of traditional algae cultivation systems (Braud and Amat 1996).

In integrated fish/seaweed aquaculture, fish respiration increases the DIC pool concentration of the effluents, providing the seaweeds with a supplementary source of DIC for their photosynthesis. Whereas the benefits of the extra nitrogen and phosphorous sources from fish excretions for algae production are well described in the literature (reviewed by Neori et al. 2004), no research has addressed if the extra DIC in the fishpond effluents is enough to maximize the biomass production in integrated aquaculture. This evaluation has to consider the physiology of the species to be cultured in terms of carbon requirements. All seaweeds use CO_2, which is fixed by Rubisco in the chloroplasts (Falkowski and Raven 1997). While some species seem to be restricted to the passive diffusion of dissolved CO_2 for photosynthesis, others have developed mechanisms to use the most abundant DIC form in the medium (HCO_3^-) as an alternative source of carbon (Beer 1994, Larsson and Axelsson

1999). No studies described so far if the tetrasporophyte phases of *Asparagopsis* spp. have mechanisms to use HCO_3^- as an alternative source of carbon for photosynthesis.

Temperature

Temperature is a very difficult/expensive parameter to control, especially in open flow-through cultivation systems. For locations where temperature varies markedly along the year, it may be useful to understand the temperature effects on the species photosynthetic performance, as it may allow us to predict the seasonal biomass production responses or production crashes in the outdoor cultivation system.

Biomass valorisation

Changes in the environmental conditions in the cultures will probably also alter the concentrations of the secondary metabolites of interest (Hay 1996). It would be important to assess if that happens with the volatile halogenated compounds (VHCs) in the cultured biomass and to understand how it is possible to induce their production to further valorise the produced biomass. According to the carbon–nutrient balance (CNB) hypothesis, the concentration of secondary metabolites in plant tissues is expected to vary with the availability of carbon and nitrogen in the environment (Bryant et al. 1983). This hypothesis predicts that under enhanced nutrient availability, the C/N ratios and the carbon based defences are expected to decrease. Additionally, most of the plant defence models for both terrestrial and marine ecology assume a cost of secondary metabolites production as reduced growth (Herms and Mattson 1992, Strauss et al. 2002, Stamp 2003). It is expected that the

manipulation of both TAN and CO_2 to the cultures will affect the C/N tissue ratio and the biomass growth, which theoretically will affect the internal levels of the VHCs in the biomass.

Understanding the physiological mechanisms behind the production of the VHCs may help us to determine how to increase their internal levels. So far, the production mechanisms of the vast number of VHCs by algae are still elusive, but at least for the major compounds (such as bromoform) they are thought to be related with oxidative stress, involving hydrogen peroxide (H_2O_2) and the haloperoxidase enzyme. The enzyme catalyzes the oxidation of halides ions (X^-: iodide, bromide and chloride) by H_2O_2, resulting in the halogenation of certain organic substrates (Butler and Walker 1993):

$$X^- + H_2O_2 + R\text{-}H + H^+ \rightarrow R\text{-}X + 2\ H_2O$$

The addition of H_2O_2 to the medium was shown to be the most important rate-determining step for the haloperoxidase activity and some of the VHCs production (Wever et al. 1991, Collén et al. 1994, Sundström et al. 1996, Pedersén et al. 1996, Manley and Barbero 2001, Ohsawa et al. 2001).

Thesis aims

This study aims to establish the tank domestication of *Asparagopsis* species; compare their nutrient biofiltration and biomass production performance with the *Ulva spp* biofilters; and study the effects of some abiotic conditions and cultivation parameters on the species photosynthesis, biomass growth and major volatile halogenated metabolites content. I firstly focused on *A. armata*, the main *Asparagopsis* species present on the Portuguese coast. Later in the thesis, the same methodologies were used to establish the cultivation of *A. taxiformis*.

Chapter 2 confirms the feasibility of integrating a land-based mass production system of *Asparagopsis armata* on a commercial fish farm considering both nutrient biofiltration and biomass production. The effects of several biomass densities on the weekly biomass yield and the effects of total ammonia nitrogen supply (TAN flux) on biomass yield, TAN removal capacity and efficiency were assessed.

In chapter 3, the species' photosynthetic responses to light and temperature are assessed, both in the laboratory and in the cultivation system. *A. armata's* photosynthetic light response under different temperatures were studied in laboratory, whereas the effects of the tank culture biomass density and high irradiance exposure on the species photoinhibition were tested in the cultivation system.

Chapter 4 aims to assess the relative performance of *A. armata* and *Ulva rigida* as biofilters of fish farm effluents. Both species were cultivated at the same time and under the same conditions to confirm early suspicions that *A. armata* is a much more efficient biofilter than the *Ulva* spp. biofilter, after comparing literature data (chapter 2). The effects of different water renewal rates on the biomass yield, N-content and TAN removal of both species were tested.

Chapter 5 asks whether fish effluents provide enough carbon to maximize *A. armata* production in culture. This was done by characterizing the DIC forms available in the water, before and after passing the fish and seaweed cultivation units and by assessing the relationship between *A. armata* photosynthesis and both DIC concentration and pH values. It was also inferred the presence of a CA-mediated mechanism and its operation conditions in *A. armata* under at the different pH cultivation conditions.

Chapter 6 establishes the conditions to cultivate in tanks of the tetrasporophyte phases of *Asparagopsis taxiformis* and *Bonnemaisonia hamifera*. Their seasonal biomass production rates were compared with *A. armata*, which was cultivated at the same time and conditions

and simultaneously, the TAN and DIC conditions that maximize *A. taxiformis* biomass production were determined.

Chapter 7 asks if and how it is possible to increase the major halogenated metabolites (bromoform and dibromoacetic acid) in *A. taxiformis* tissue. Both metabolites internal levels were monitored in individuals cultivated in enriched hydrogen peroxide (H_2O_2) mediums and cultivated at different levels of TAN and CO_2 in laboratory and in the outdoor cultivation system.

CHAPTER 2

The tetrasporophyte of *Asparagopsis armata* as a novel seaweed biofilter *

Abstract

The red seaweed *Asparagopsis armata* (Harvey; Rhodophytae, Bonnemaisoniaceae) produces biologically active secondary metabolites that are valuable natural ingredients for cosmetics and medicine and its cultivation may therefore be a profitable venture. The tetrasporophyte of this species (*Falkenbergia rufolanosa*) was successfully tank-cultivated as a continuous biofilter for the effluent of a commercial fish farm in southern Portugal. Optimal stocking density for highest biomass yield and a low level of other algal species in winter and late spring was 5 g centrifuged fresh weight L^{-1}. The effect of total ammonia nitrogen supply (TAN flux) on biofiltration and biomass yield was investigated in winter and spring. Results revealed that *A. armata* is currently the seaweed-biofilter with the highest TAN removal of up to 90 μmol L^{-1} h^{-1} at a TAN flux of about 500 μmol L^{-1} h^{-1}. In the tanks used, this is equivalent to a removal of up to 14.5 g TAN m^{-2} d^{-1}. At a lower TAN flux of about 40 μmol L^{-1} h^{-1}, TAN removal by *A. armata* is more than double to what is reported at this flux for another successful seaweed biofilter, the genus *Ulva*. Monthly variation of *A. armata* biomass yield peaked in May and was lowest in January. At TAN fluxes between

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300 and 400 µmol L^{-1} h^{-1}, an average water temperature of 21.7 °C and a total daily photon flux density of 47 mol m^{-2}, seaweed yield was over 100 g DW m^{-2} d^{-1} with a recorded maximum of 119 g. During spring, autumn and early summer, the biomass of *A. armata* within the experimental tanks doubled every week. A model for the up scaling of this finfish integrated aquaculture of *A. armata* varies the investment in biofilter surface area and estimates the return in biofiltration and biomass yield. Highest TAN removal efficiencies will only be possible at low TAN fluxes and a very large biofilter area, resulting in a low production of biomass per unit area. To remove 50 % of TAN from the effluent (1 mt *Sparus aurata*; 21 °C), 28 m^2 of biofilter, designed to support a water turnover rate of 0.8 Vol h^{-1} would be necessary. This system produces 6.1 kg FW (1.5 kg DW) of *A. armata* per day and has the potential to turn biofiltration into an economically sustained, beneficial side effect.

Introduction

Seaweed biofilters are important elements in many proposed integrated or multi trophic level mariculture systems. Yet, there are only few successfully tested species out there and little information on economic viability as biofilters is available (Neori et al. 2004, Troell et al. 2003 - and references within both). As a general principle, and to make operation financially worthwhile, the biofilter species itself should be economically interesting. Unfortunately, macroalgae with an existing high market value, such as the genus *Gracilaria*, are highly susceptible to epiphytism when grown under a continuous supply of aquaculture effluent (Friedlander et al. 1987, 2001, Haglund and Pedersén 1993). As a result, their use as biofilters is rather limited (Buschmann et al. 1994, Neori et al. 2000, 2004).

This article reports on the successful establishment of a novel and commercially diverse species as a seaweed biofilter for mariculture effluent. It is the filamentous tetrasporophyte of the red seaweed *Asparagopsis armata* (Harvey) that is also referred to as *Falkenbergia rufolanosa.* The *Falkenbergia*-phase occurs naturally as a free-floating, small "pompon" and this is an ideal morphology for tank cultivation. Among other species of the order Bonnemaisonales, *A. armata* produces high levels of halogenated, biologically active, secondary metabolites (McConnell and Fenical 1977, 1980). These compounds are natural antibiotic substances that act as chemical defence against grazers and epibiota and may be marketable for a wide range of natural applications in antifouling, as preserving agents and in cosmetics or medicine (Codomier et al. 1981, de Nys et al. 1995, Steinberg et al. 2001, and references in all).

The presented research focuses on the feasibility of integrating a land-based mass production system of *A. armata* on a commercial fish farm, with the objectives of investigating both biofiltration and seaweed production. To optimize cultivation conditions,

the effects of culture density on biomass yield and the effects of total ammonia nitrogen supply (TAN flux) on biomass yield, TAN removal and removal efficiency were tested. A model for the up scaling of this cultivation system to biofilter the effluent generated by one metric ton of commercially raised Gilthead Seabream (*Sparus aurata*) was developed. This model varies the investment in biofilter area and estimates return in the form of produced seaweed biomass and removed TAN.

Material and Methods

Experimentation took place at Aquamarim Lda., a land-based, semi-intensive fish farm in southern Portugal with an annual production of 40 mt of *Sparus aurata*. Turbid wastewater from the effluent channel of the farm was screened for larger particles with an automatic cartridge filter (150µm; Amiad Ltd, Israel). This water was then continuously supplied to 12 experimental and cylindrical white (light transparency ~70%) polyethylene tanks (Allibert Buckhorn C1100; 110 L capacity). The tanks were arranged next to each other in two rows, which partly shaded the sidewalls. Each tank had a footprint area of 0.23 m^2 and a water depth of 0.48 m. To avoid the loss of biomass in these flow-through units, overflows along the water surface were equipped with cylindrical 0.5 mm net screens. Lastly, continuous aeration, supplied through a circular ring that was placed along the edge of the tank bottom, fluidized the unattached seaweed. A probe (YSI 6600; YSI, USA) submerged in one of the tanks logged the water temperature while a Li-190SA Quantum Sensor installed 2.5 m above the tanks that connected to a Li-1000 Data Logger (both LI-COR, Inc. USA), monitored open-air photon flux density (PFD). An initial sample of *A. armata*'s free-floating *Falkenbergia*-phase (30g fresh weight) was collected at the coastline

of southern Portugal (37° 00.0' N; 007° 55.5' W), identified using appropriate taxonomic keys (Dixon and Irvine, 1977) and cultivated in the tanks.

The relationship between stocking density of *A. armata* and weekly production was determined in two periods, November/December 2002 and June 2003. In winter, a wide range of stocking densities from 0.8 g centrifuged fresh weight (FW) L^{-1} to 9 g FW L^{-1} were tested haphazardly along the sampling period, in order to reveal both the general response pattern of the relationship between density and yield and the density that produced the highest yield. Care was taken to maintain the same water exchange rates (2 Vol h^{-1}) and therefore the same weekly nutrient flux for all tanks. The biomass yield of *A. armata* was established once per week by harvesting each tank with mesh bags (0.1 mm mesh) and draining the biomass to constant fresh weight at 2800 rpm in a standard domestic centrifuge. Yields (Y) were calculated from the equation Y (g DW m^{-2} wk^{-1}) = (N_t - N_0) / t / (DW / FW) / A (modified from DeBoer and Ryther, 1977), where N_t is the final fresh weight, N_0 the initial fresh weight, DW/FW the dry weight / centrifuged fresh weight ratio and A the area covered by the cultivation tank in m^2. To determine DW/FW, centrifuged fresh seaweed samples of 10 g were oven-dried (48 h; 60 °C) and weighed after cooling down in a Silica-desiccator.

In late spring, when irradiance levels are higher, an experiment was designed to test if the optimal stocking density would be more elevated. The effects of density on yield were tested in replicate tanks (n=2) in two consecutive weeks. Tanks were stocked at 4, 5, 6, 7, 8 and 9 g FW L^{-1} and the yield of *A. armata* was measured as described above. A two way ANOVA was performed to test for the effects of time and density on yield. As the effects of time were not significant, a one way ANOVA was subsequently done, considering 4 replicates for each density to test for significant differences between different stocking densities. When significant differences were found ($p < 0.05$), a Tukey HSD test was applied to test for significant differences in factor levels ($p < 0.05$).

The monthly variation of yield at the optimum stocking density (5 g FW L^{-1}), revealed in the above experiments, was recorded from October 2002 to July 2003. The yields were recorded weekly in two tanks. The monthly average was computed using the 8 available measurements. To avoid pseudoreplication, the algae from all the tanks were always mixed before re-stocking to individual tanks and all water exchange rates were maintained at 2 Vol h^{-1}.

TAN flux effects

The amount of ammonium that is available to the seaweeds within a tank is dependent on the ammonium concentration of the incoming water from the fishponds and on the water renewal of the tank. Accordingly, TAN flux was calculated as the product between TAN concentration of the incoming water to a tank (µmol L^{-1}) and the water renewal rate of the tank (number of volumes h^{-1}).

In January and May 2003, two week long nutrient flux experiments tested the effects of total ammonia nitrogen (TAN = $NH_4^+ + NH_3$) flux on *A. armata*'s biofiltration and yield. Tanks, stocked at the previously determined optimal density, were kept at various, manually adjusted and carefully monitored water flow rates. In January, the water exchange levels were chosen to create a range of TAN fluxes similar to the ones reported in experimentation on *Ulva* (Cohen and Neori 1991, Jiménez del Rio et al. 1994, Mata and Santos 2003), but these proved to be limiting for *A. armata*. In May, tanks were therefore supplied with higher fluxes. During both trials, a closely monitored nutrient removal experiment was done on day two in week two. To assess biofilter performance in the dark, the first and the last measurements were done at night. Water pH and DO were measured every three hours in every tank and the fishpond effluent (OxyGuard probe). Water samples (n = 3) were also

taken every three hours and passed through sterile 0.25 µm filters (CF Whatman). The TAN concentration in these samples was analysed the following day in accordance with Grasshoff et al. (1983).

Biofiltration of *A. armata* was subsequently presented in two ways; the TAN removal rate (µmol TAN L^{-1} h^{-1}) and the TAN removal efficiency (%). Removal rate in each tank was calculated as the difference between influent and effluent TAN flux and removal efficiency as the missing ratio between effluent and the influent TAN flux. It was decided to present the removal rate per litre of seaweed tank and not per m^2 tank area because it would otherwise vary with tank depth, making an extrapolation for other systems more complicated. Michaelis-Menten curves (Hyperbola, single rectangular, 2 parameters; $y = ax / (b + x)$) were fitted to plots of TAN flux versus removal rate data to allow an estimation of maximum TAN removal ($a = V_{max}$) and half saturation constants ($b = K_s$). To better illustrate TAN removal efficiency, exponential decay curves were plotted against data points of TAN flux versus removal efficiency.

To illustrate the effects of TAN flux on the production of *A. armata*, weekly seaweed yield in each tank was plotted against the respective 24 h average TAN flux (µmol L^{-1} h^{-1}) obtained on the day of water analysis. This plot was therefore based on the assumption that the daily average concentration of TAN in the fishpond effluent did not vary significantly along the different days of the experimental period. Tissue C and N content was analysed using a CHNS-analyser (Carlo Erba CHNS-O EA1108). An estimate of total weekly nitrogen uptake by *A. armata* (N-yield) was made by multiplying *A. armata* biomass yield during the second week with N content of seaweed tissue (protein content). This N-yield was then compared with TAN removal data derived from water analysis.

Results

Throughout daylight hours, water pH and DO levels in the seaweed tanks were higher than in the supplied fishpond effluent and this difference was more pronounced in spring than in winter. In the fishpond effluent, levels of pH decreased from about 8.2 in the morning to afternoon levels of 7.7 in winter and 7.5 in spring. Likewise, DO-levels decreased from 9.7 mg L^{-1} to 9.1 mg L^{-1} in winter and 8.7 mg L^{-1} in spring. Contrary to this, and more so inside seaweed tanks with a low water exchange rate, pH increased at 1:00 pm to maxima of 8.8 in winter and 9.1 in spring. Following the same pattern, DO in the tanks increased to supersaturated levels reaching maxima of 14 mg L^{-1} in winter and 16 mg L^{-1} in spring. Generally, pH and DO in seaweed tanks with higher water exchange rates remained closer to levels of the fishpond effluent. At night and in both seasons, no differences in water pH and DO levels were detected between the in- and effluent of all seaweed tanks.

In winter, the production of *A. armata* peaked at about 400 g DW m^2 wk^{-1}, when stocked at a density around 5 g FW L^{-1}, whereas in the spring the peak was at about 700 g DW m^2 wk^{-1} at the same density (Fig. 1). At densities lower than 5 g FW L^{-1}, the cultures of *A. armata* were often invaded by nuisance species such as *Ectocarpus* and *Ulva*. When grown at and above 5 g L^{-1}, the levels of these species always remained low. For these two reasons 5 g L^{-1} was selected as the optimal stocking density for all subsequent experiments. In the density experiment in June, 5 g FW L^{-1} were also optimal (Fig. 1) and the level of stocking density had a significant influence on yield ($p < 0.001$). The following Tukey HSD test revealed significant differences in the yield between all stocking densities ($p < 0.001$). The DW/FW-ratio of *A. armata* was constant in both seasons and at all tested densities and around 0.25±0.01 (average ± standard deviation).

The monthly variation of biomass yield at optimal stocking density and a water exchange of 2 Vol h^{-1} followed temperature variation and photon flux density (Fig. 2) but it was impossible to separate the relative effect of these two factors. Biomass yield was reduced in January, when temperature and light levels were among the lowest, and peaked in May when both light and temperature reached higher and more optimal levels. In June and July, when daily highest temperatures reached 28.4 ºC, the yield dropped. The average yield over the whole ten months of cultivation was 446 g DW m^{-2} wk^{-1}.

TAN flux effects

In January, the concentration of nutrients in the fish farm effluent increased only slightly during the day and the 24 h average of TAN concentrations was 26.2±3.2 µmol L^{-1}. In May, TAN concentrations in the fishpond effluent were a) considerably higher than in January and b) doubled from 85 µmol L^{-1} at 6:00 am to 167 µmol L^{-1} at 9:00 pm. As a result, TAN flux in May at a comparable water exchange rate as in January was higher and increased throughout the day.

In both flux experiments (Fig. 3 and 4), TAN removal increased with TAN flux and followed Michaelis-Menten kinetics, while the respective removal efficiency was inversely related to TAN flux. The TAN removal rate in January, expressed by V_{max} and K_s, was one order of magnitude lower than in May. This was probably a direct consequence of the TAN concentration and the range of TAN fluxes tested, which were much lower in January than in May. However, for the same TAN flux of 35 µmol L^{-1} h^{-1}, the January removal rate was 7 µmol L^{-1} h^{-1}, while in May it reached 30 µmol L^{-1} h^{-1}, suggesting a seasonal effect on this relationship (Fig. 3 and 4; all times). At this flux, removal efficiency in January (20%) was consequently much lower than in May (85%). Even though *A. armata* biofiltration in both

seasons was higher during the day than at night (Fig. 3 and 4), data revealed that biofiltration without light was relevant. The maximum TAN removal rates (V_{max}) in the dark were about half of those during daytime.

The biomass yield of *A. armata* was also strongly influenced by TAN-flux and season (Fig. 5). When comparing winter with spring, the biomass yield versus TAN flux curve saturated at lower fluxes in January than in May. Maximum yield recorded in May was 839 g DW m^{-2} wk^{-1}. The elemental carbon and nitrogen content of the seaweed tissue did not show a significant variation either with nutrient flux or season. The average C content was 35.5 % and the N content was 6.5 %. The effects of TAN flux on both the weekly total nitrogen uptake by *A. armata* (N-yield) and on the TAN removal are shown in Figure 6. As expected, both variables increased with TAN flux. However, N-yield values were considerably higher than TAN removal values in January, while in May the opposite was observed.

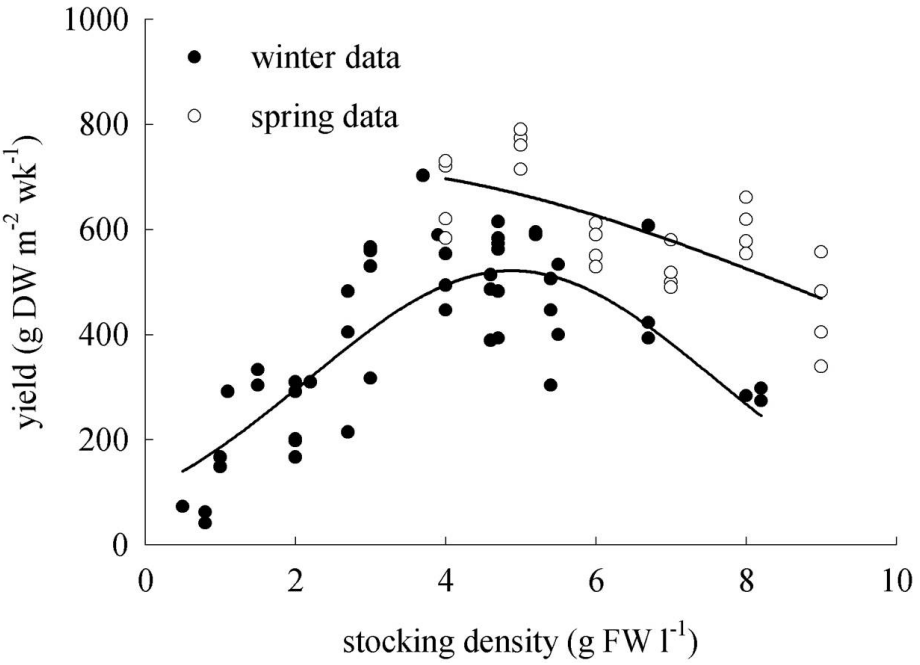

Fig. 1. Relationships between stocking density and biomass yield of *Asparagopsis armata*, in winter (●) and spring (○). Each data point represents weekly seaweed yield in an individual tank stocked at the indicated density. All tanks were maintained at the same water exchange rate (2 Vol h^{-1}).

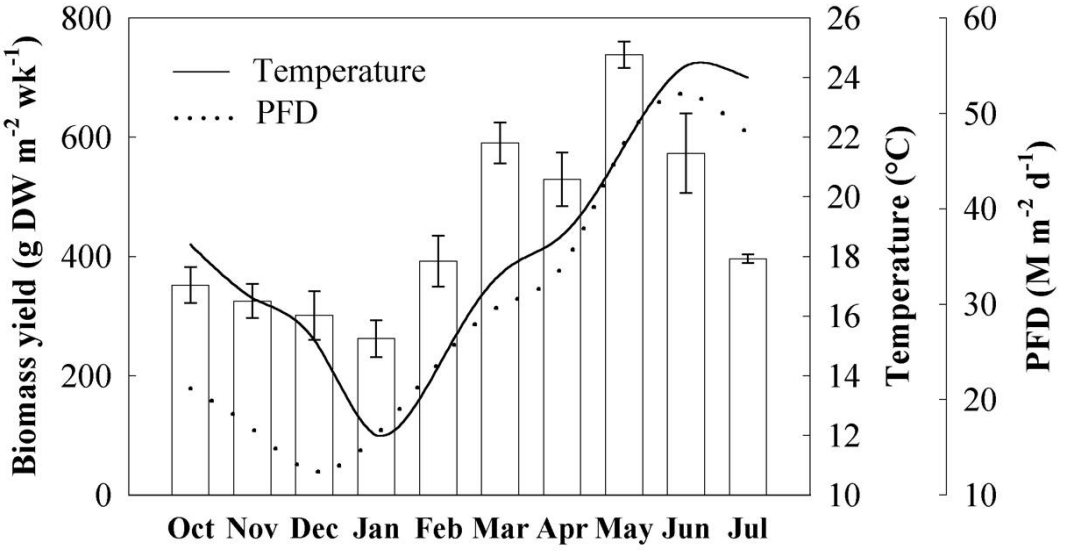

Fig. 2. Monthly variation of biomass yield of *Asparagopsis armata* at optimal stocking density (5 g FW L^{-1}). All tanks were maintained at the same water exchange rate (2 Vol h^{-1}). The lines represent the monthly averages of water temperature (continuous line) and daily photon flux density (PFD; dotted line).

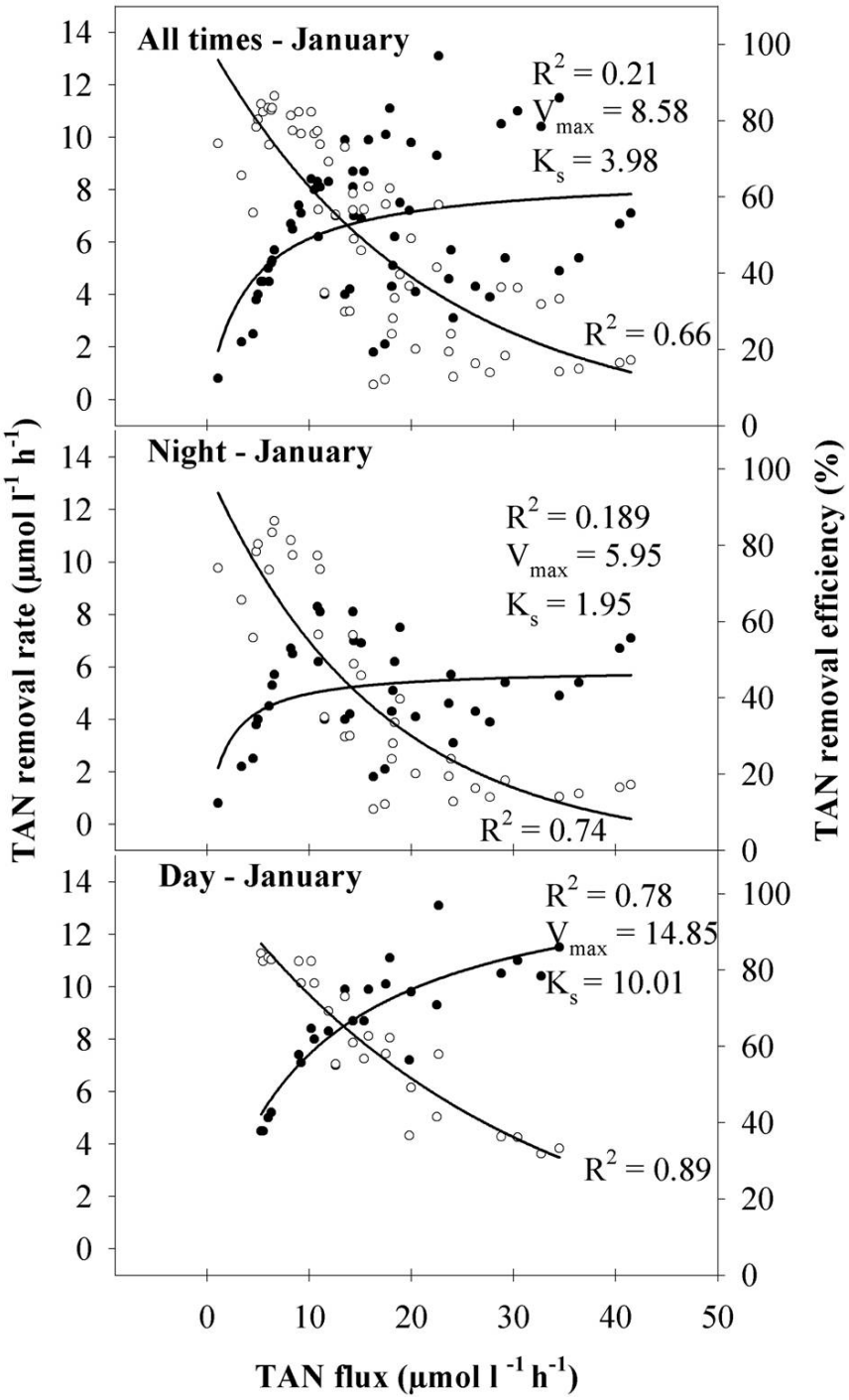

Fig. 3. Effects of TAN flux on TAN removal rate (●) and TAN removal efficiency (○) of *Asparagopsis armata* in January. Top TAN fluxes reach 40 µmol L^{-1} h^{-1}. All tanks were stocked with 5 g FW L^{-1}.

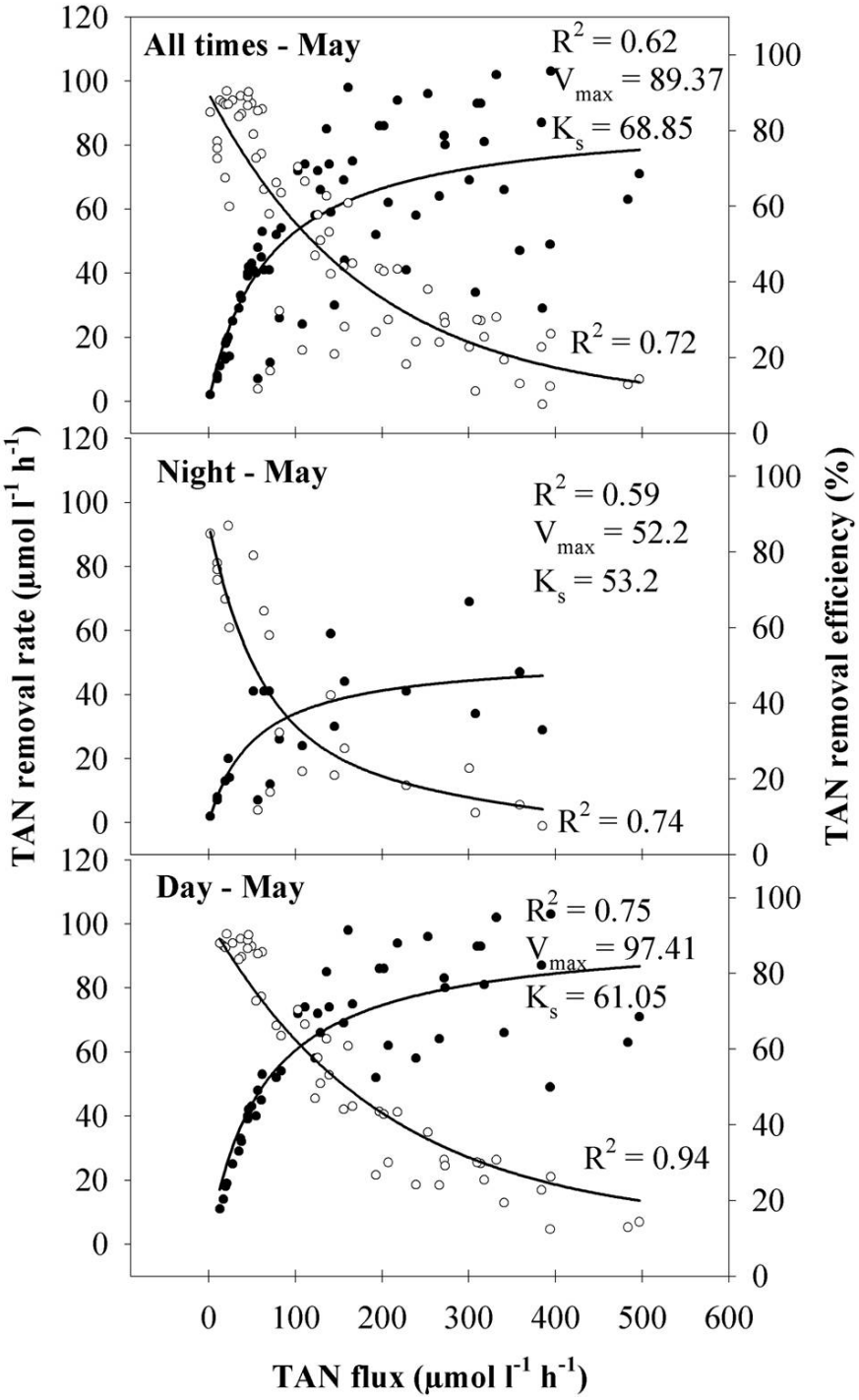

Fig. 4. Effects of TAN flux on TAN removal rate (●) and TAN removal efficiency (○) of *Asparagopsis armata* in May. Top TAN fluxes reach 500 µmol L^{-1} h^{-1} and are one order of magnitude higher than in January (Fig. 3). All tanks were stocked with 5 g FW L^{-1}.

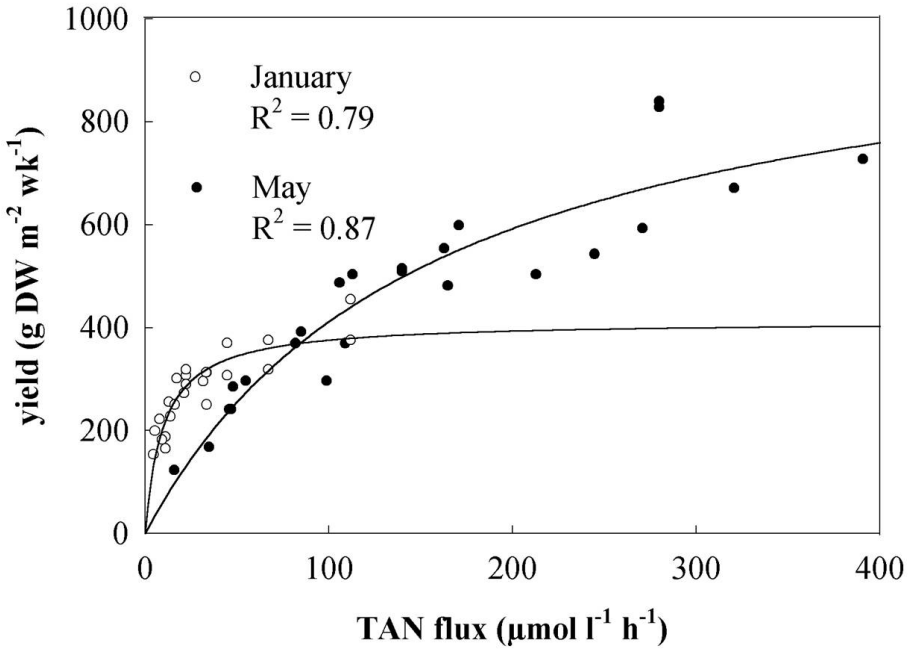

Fig. 5. Weekly yield of *Asparagopsis armata* in January and May's flux experiments plotted against TAN flux (two weeks; number of individual tanks: 24).

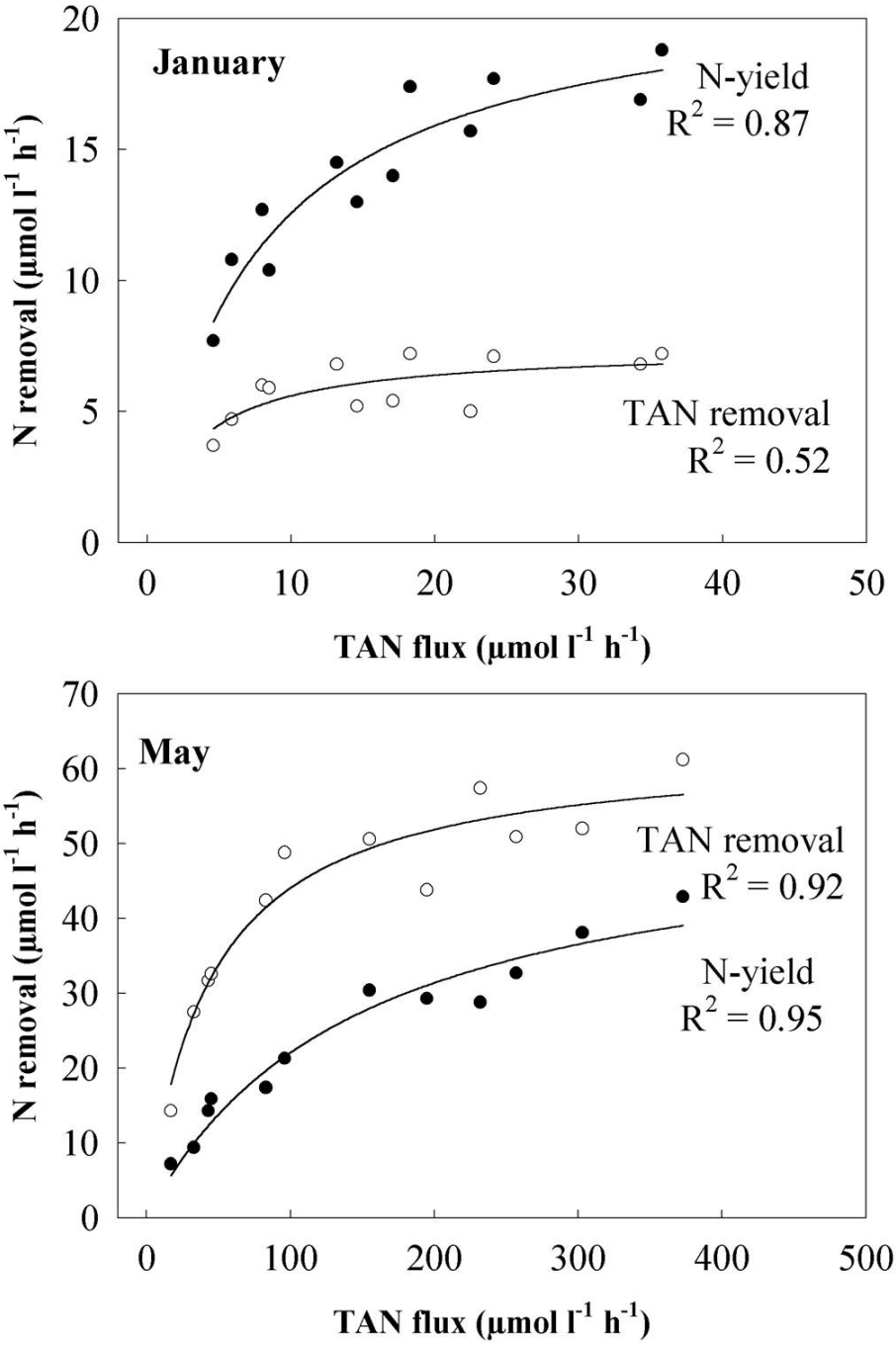

Fig. 6. Relationship between *Asparagopsis armata* N-yield of produced biomass (●) and 24 h TAN-removal (○) plotted against TAN fluxes. Data presented here are from the second week of both nutrient flux experiments when water analysis for TAN was done and seaweed tissue N-content was analysed (12 individual tanks each). Due to the range of TAN fluxes, the scales for January and May differ.

Table 1. Comparison of relevant biofiltration data of the genus *Ulva* with *Asparagopsis armata* (*Falkenbergia* phase).

Species	Tank volume L	Stocking density g FW L^{-1}	Mean water temperature °C	Maximum water exchange Vol h^{-1}	TAN flux µmol L^{-1} h^{-1}	TAN removal µmol L^{-1} h^{-1}	Removal efficiency %	Biomass yield g DW m^{-2} d^{-1}	reference
Ulva lactuca	600	1.7	20	0.5	40	23	40	55	Cohen and Neori 1991, Neori et al. 1991
Ulva rigida	750	2.5	24	0.5	46 [1]	18	33	40	Jiménez del Rio et al. 1994, 1996
Ulva rotundata	1900 [2]	2	22	0.6	18	10	60	48	Mata and Santos 2003
A. armata	110	5	13	1.3	35	12	34	43	This chapter
A. armata	110	5	22	3.0	500	100	18	120	This chapter
A. armata	110	5	22	0.4	40	35	86	40	This chapter

[1] reported as the total of dissolved inorganic nitrogen which includes small amounts of nitrite and nitrate; [2] raceway

Table 2. A model for the performance of an integrated aquaculture system of *Asparagopsis armata* (water depth 48 cm; water temperature 21 °C).

Biofilter area (m^2)	4	8	16	28	58	100	300
TAN flux (µmol L^{-1} h^{-1})	775	388	194	111	53	31	10
Production (g FW m^{-2} d^{-1})	465	391	297	219	131	84	31
Total production (g FW d^{-1})	1859	3130	4757	6119	7625	8440	9360
TAN removal (%)	1.0	9.8	30.6	50.0	70.1	80.0	90.3
TAN removal (g)	5	49	153	250	350	400	452
Water turnover (Vol h^{-1})	5.5	2.8	1.4	0.8	0.4	0.2	0.1

The investment in biofilter area treating the daily effluent generated by 1 mt *Sparus aurata* determines the return in biofiltration and biomass production.

Discussion

An important variable to control in the integrated cultivation of *Asparagopsis armata* is the optimal stocking density, which - in the tanks used - was found to be 5 g FW L^{-1} (2.4 kg m^{-2}). Lower density cultures were frequently invaded by nuisance species and either lower or higher densities than 5 g FW L^{-1} led to a decrease in biomass production. Optimal stocking density values determined for *A. armata* were similar to values found for other tank-cultivated red species like *Gracilaria* (Capo et al. 1999) and *Palmaria* (Demetropoulos and Langdon 2004) and much higher than for the green species *Ulva* (Cohen and Neori 1991, Mata and Santos 2003). This is probably related to the lower light requirements of the red seaweed species tested, compared to *Ulva*.

Our results reveal that *A. armata* is currently the seaweed-biofilter with the highest reported TAN removal in integrated aquaculture (Table 1) with up to 90 µmol L^{-1} h^{-1} at a TAN flux of about 500 µmol L^{-1} h^{-1}. This is far above the highest values previously described for the biofilter genus *Ulva*. Yet, the corresponding authors have not experimented with similarly high TAN fluxes (Table 1). This could mean that other seaweed biofilters may also have greater potential than previously thought. However, a direct comparison with other species at the same levels of TAN flux and temperature further support the previous claim. At TAN fluxes of about 40 µmol L^{-1} h^{-1}, *A. armata* removed 35 µmol L^{-1} h^{-1} while *Ulva* is reported to only remove up to 16 µmol L^{-1} h^{-1}. Moreover, *A. armata* is a very effective biofilter even at a rather low temperature. In winter, at temperatures around 13 ºC and a TAN flux of 35 µmol L^{-1} h^{-1}, *A. armata* removed about 12 µmol L^{-1} h^{-1} and this is an important figure when compared with the genus *Ulva*, which removes this level of TAN at temperatures of around 20 ºC (Table 1). In addition, *A. armata*'s removal rate under these

conditions would have increased further if higher TAN concentrations and fluxes had been available (Fig. 3).

Care must be taken in interpreting TAN removal rates measured by water analysis as they do not only represent seaweed uptake but also other forms of removal from the cultivation system, which may include processes such as nitrification, denitrification and ammonia volatilization. The differences found between TAN removal and N-yield (Fig. 6) may reflect this issue. In May, the N-yield was lower than TAN removal suggesting the effect of such factors. On the contrary, in January N-yield was higher than TAN removal, suggesting that *A. armata* might have been TAN limited and because of this, also taken up nitrate. Indeed, the plateau of the Michaelis-Menten curve at daytime in January (Fig. 3) is less evident than of all other curves obtained. Another likely reason for these discrepancies may be that the TAN removal values are calculated from one day's average whereas the N-yield values represent the average weekly measurement. The environmental conditions of the day when TAN removal was measured might not represent the whole week conditions. In fact, the weekly averages of irradiance (197 μmol m^{-2} s^{-1}) and water temperature (14.2±2.7 °C) in January were higher than on the day of TAN removal assessment through water analysis (169 μmol m^{-2} s^{-1} and 12.8±1.3 °C). The N-yield might therefore be overestimated in relation to TAN removal, which was measured on the second day of the week. On the contrary, in May, the mean irradiance of the whole week was lower (607 μmol m^{-2} s^{-1}) than the irradiance of the day of water analysis (658 μmol m^{-2} s^{-1}), underestimating N-yield in relation to TAN removal. Moreover, such daily variation probably not only had a direct influence on the seaweed productivity but also changed the TAN concentration in the fishpond effluent and overall nutrient availability to the seaweed by altering the feeding rate and ammonia excretion of the fish.

An important aspect of integrated seaweed aquaculture for biofiltration is that the system with *A. armata* also removes TAN with no light available for photosynthesis. Both *A. armata* (Fig. 3; night data) and *Ulva* (Mata and Santos 2003, Schuenhoff et al. 2003) showed significant night removal rates around 6 μmol L^{-1} h^{-1}. Again, removal rates reached with *A. armata* in January could have been higher at an increased supply of TAN. In May, when TAN was not limiting and temperature was optimal, an unprecedented removal of 50 μmol TAN L^{-1} h^{-1} was obtained at night (Fig. 4; night data).

During spring, autumn and early summer, the biomass of *A. armata* within the experimental tanks doubled every week. At TAN fluxes around 400 μmol L^{-1} h^{-1}, biomass yield was over 100 g DW m^{-2} d^{-1} (Fig. 5). Contrary to the expected, at identical TAN fluxes and water temperatures, the DW yield of *A. armata* was not higher than of *Ulva* (Table 1). The higher biofiltration is explained by the 60 % higher N-content of *A. armata* compared to *Ulva* (Cohen and Neori 1991, Mata and Santos 2003, Schuenhoff et al. 2003).

Curiously, the biomass yield of *A. armata* at low TAN flux in January was higher than in May (Fig. 5). The concentration of TAN in the fishpond effluent was lower in January and thus increased water exchange rates were necessary to achieve comparable TAN-fluxes. This water supplied more dissolved inorganic carbon (DIC) for photosynthesis, and consequently for growth. Dissolved inorganic carbon has been referred to as being the main limiting factor for photosynthesis of tank-cultivated seaweed (Bidwell and McLachlan 1985, Blakeslee 1986, Hanisak 1987) and is often artificially added to the cultivation medium at high financial cost. Compared to natural seawater, the concentration of DIC in fishpond effluent is higher due to fish respiration and the heterotrophic breakdown of dissolved organic substances in the fishponds (Krom and Neori 1989) and thus the water renewal rate will have an important effect on the carbon availability for the seaweed. Indeed, the measured water pH increase in the seaweed tanks during daylight hours shows the effects of photosynthesis.

Higher midday maxima in spring may depend on an increased carbon utilisation during stronger light levels. To achieve elevated biomass yields, water exchange rates should therefore not limit the availability of both carbon and nitrogen to seaweed growth. The drawback is that biofiltration efficiency (%) will decrease. The optimization of an integrated aquaculture system will thus depend on the objectives to achieve, i.e. biofiltration versus biomass yield. Alternatively, it is possible to design a several-stage biofilter that incorporates a logic found to optimise both seaweed production and nutrient removal efficiency (Neori et al. 2003, Schuenhoff et al. 2003).

A model for up scaling the biofilter

In a fishpond, food input and water exchange generally determine effluent TAN concentration. Assuming a properly managed aquaculture, where these factors are monitored, it is therefore possible to estimate approximate TAN concentration by simple budget calculation. The nutrient flux to a seaweed biofilter and the resulting level of biomass yield could therefore be controlled without expensive and time-consuming water analysis. At a water temperature of 21 °C, 1 mt of commercially fed, adult *Sparus aurata* generate roughly 500 g TAN d^{-1} (Lupatsch and Kissil 1998, Lupatsch et al. 2003). In addition and due to the toxicity of the compound, water exchange should maintain safe conditions for *S. aurata* with TAN-levels below 2 mg L^{-1} (Porter et al. 1987). On a simplified 24 h level and per mt of fish, this demands a daily water exchange of 250 m^3, creating an effluent with a TAN concentration of 140 µmol L^{-1}. Using these conditions, we have calculated the biofiltration and biomass yield of *A. armata* when grown in a tank system with a wide range of cultivation areas (Table 2). It becomes apparent that top TAN removal efficiencies will only be possible at low TAN fluxes and a very large biofilter area, resulting in a low

production of biomass and reduced economic viability (see Troell et al. 2003). To remove 50 % of TAN from the effluent (1 mt *S. aurata*; 21°C), 28 m^2 of biofilter, designed to support a water turnover rate of 0.8 Vol h^{-1} would be necessary. The daily production of *A. armata* would be 6.1 kg FW (1.5 kg DW). This model is based on the TAN removal rate kinetics of the 24h-performance of *A. armata* at 21°C, measured in May (Fig. 3; all times). At lower temperatures and irradiance in winter, seaweed growth and biofiltration will be reduced. Yet, this effect will be buffered by similarly reduced food consumption and TAN excretion of *S. aurata* and a similar biofilter area per mt of fish will therefore be adequate in both seasons.

An integrated aquaculture system of *A. armata* in southern Portugal will maintain high production levels during most of the year as even temperatures in winter are never too low (Fig. 2). The species lethal limits are 5 - 27°C (Orfanidis 1991) and the optimal range for growth is 10 – 21 ºC (Oza 1989, Orfanidis 1991). The critical period is during summer, when daily average levels should not exceed these limits. During June and July, at a daily average temperature of 24 ºC, the biomass yield decreased (Fig. 2) and due to reduced growth of *A. armata*, other opportunistic species took over the culture.

To optimise large-scale production, *A. armata* should be cultivated in bigger tanks than the ones used here. However, an increased tank-size may become a potential bottleneck for light and the rapid water turnover rates necessary for high biomass yield. It is therefore important to pay special attention to the engineering of such larger production systems with regard to depth, properly and correctly scaling and positioning outflow screens, water inlets and aeration tubes and also designing an efficient system for harvests. Production costs of *A. armata* will depend on these factors. Market value and demand for the produced biomass cannot be estimated but will eventually depend on the range of developed applications. Moreover, integrated operations such as this one may also become economically interesting by reducing potential taxes on nutrient release.

Conclusion

Results presented here confirm the discovery of easily tank-cultivated, fast-growing red seaweed that is an excellent biofilter for temperate latitudes and produces economically valuable secondary metabolites. The integrated production of marine fish and *Asparagopsis armata* has the potential to be ecologically, economically and socially more sustainable than current practice. It will reduce environmental impact of fish farming, produce an extra income for the farmer and create additional jobs while helping to improve the public image of intensive aquaculture.

CHAPTER 3

The effects of light and temperature on the photosynthesis of the *Asparagopsis armata* tetrasporophyte (*Falkenbergia rufolanosa*), cultivated in tanks *

Abstract

The integrated aquaculture of the tetrasporophyte of *Asparagopsis armata* Harvey (*Falkenbergia rufolanosa*) using fish farm effluents may be viable due to the species high capacity of removing nutrients and its content of halogenated organic compounds with applications on the pharmaceutical and chemical industries. In order to optimize the integrated aquaculture of *A. armata*, we followed the daily variation of the potential quantum yield (F_v/F_m) of PS II on plants cultivated at different biomass densities and different total ammonia nitrogen (TAN) fluxes to check if they are photoinhibited at any time of the day. As well, the photoinhibition under continuous exposure to highly saturating irradiance and its potential for subsequent recovery in the shade was assessed. The potential for year round cultivation was evaluated by measuring rates of O_2 evolution of plants acclimated at temperatures ranging from 15 to 29 ºC, the temperature range of a fish farm effluent in southern Portugal where an integrated aquaculture system of *A. armata* was constructed.

Photoinhibition does not seem to be a major constrain for the integrated aquaculture of *A. armata*. Only when cultivated at a very low density of 1.5 g fresh weight (FW) L^{-1} there

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was a midday decrease in maximal quantum yield (F_v/F_m). At densities higher than 4 g FW L^{-1} no photoinhibition was observed. When exposed to full solar irradiance for one hour, *A. armata* showed a 33% decrease in F_v/F_m, recovering to 86% of the initial value after two hours in the shade. A midday decline of the *A. armata* Fv/Fm was also observed under the lowest TAN flux tested (~6 µM h^{-1}), suggesting that this fast and easy measurement of fluorescence may be used as a convenient diagnostic tool to detect nutrient-starved unbalance conditions of the cultures. Maximum net photosynthesis peaked at 15 ºC with 9.7 mg O_2 g dry weight $(DW)^{-1}$ h^{-1} and remained high until 24 ºC. At 29 ºC, the net oxygen production was significantly reduced due to a dramatic increase of respiration, suggesting this to be the species' lethal temperature threshold.

Results indicate that the tetrasporophyte phase of *Asparagopsis armata* has a considerable photosynthetic plasticity and confirm it as a good candidate for integrated aquaculture at temperatures up to 24º C and cultivation densities of at least 5 g FW L^{-1}. When cultivated at these densities, light does not penetrate below the first few centimetres of the surface zone. Plants circulate within the tanks, spending around 10% of the time in the first few centimetres where they are able to use efficiently the saturating light levels without damaging their photosynthetic apparatus.

Introduction

There is an increasing interest in cultivating seaweed species that produce fine chemicals for the pharmaceutical and chemical industries. *Asparagopsis armata* Harvey, as most of the species of the Bonnemaisoniaceae family, is known to produce halogenated organic compounds with remarkable antibacterial and antifungal activity (McConnell and Fenical 1977) that can be used to obtain cosmetic and/or pharmaceutical preparations. This species also produces sulphated galactans with promising therapeutic applications (Braun et al. 1983, Caporiccio et al. 1983), and new sources of anti-HIV compounds (Haslin et al. 2001). The potential economical value of the species instigated the cultivation of its tetrasporophytic phase, commonly known as *Falkenbergia rufolanosa*, to biofilter fish farm effluents (Schuenhoff et al. 2006 - Chapter 2). *A. armata* proved to be an excellent alternative to the most frequently used macroalga in polyculture, *Ulva spp*.

Both nutrient assimilation and biomass production are temperature- and light-dependent processes. In order to study the cultivation conditions that optimize the year-round production of *A. armata* we assessed the species' photosynthetic responses to these environmental factors by O_2 evolution (P/I curves) in the laboratory and by pulse amplitude modulation (PAM) fluorescence field measurements (Schreiber et al. 1995). Short-term P/I measurements allow an estimation of temperature effects on the photosynthetic performance under saturating and sub-saturating irradiances. Such information is important for optimizing aerated tank cultivation, where the circulation pattern of plants alternately exposed them to full sunlight and darkness. At the surface, high levels of photosynthetically active radiation (PAR) may be a threat to the plant metabolism if the irradiance exceeds the demands of photosynthesis (Osmond 1994, Aguirre-von-Wobeser et al. 2000). Thus it is important to know whether the plants are capable of photoacclimation or if they are photoinhibited at any

time of the day when cultivated at different densities and nutrient fluxes. Pulse amplitude modulation (PAM) fluorescence field measurements (Schreiber et al. 1995) allow a non-intrusive assessment of the effects of stress factors such as excessive radiation.

The specific objectives of this work were (1) to test the effects of biomass density and total ammonium nitrogen (TAN) flux on photoinhibition during a daily cycle, (2) to assess photoinhibition under continuous exposure to highly saturating irradiance and the potential for subsequent recovery in the shade and (3) to assess *A. armata*'s photosynthetic light response under different temperature conditions.

Materials and methods

Seaweed cultivation conditions

This study was conducted in a *Asparagopsis*-biofilter system (Schuenhoff et al. 2006 - Chapter 2) on a *Sparus aurata* fish farm, Aquamarim, located in Ria Formosa lagoon, southern Portugal. *Asparagopsis armata* was cultivated in 110L (0.48 m * 0. 23 m^2) cylindrical white polyethylene aerated tanks that were supplied with particle screened (150µm; Amiad) fishpond effluent rich in total ammonia nitrogen (TAN = $NH_4^+ + NH_3$). Irradiances inside and outside the seaweed tanks were measured with a spherical Li-193SA Underwater Quantum Sensor and a Li-190SA Quantum Sensor respectively, both connected to a Li-1000 Data Logger (Li-Cor, Lincoln, Nebraska, USA). The light availability inside the tanks with different biomass densities was determined by measuring noontime PAR at a depth of 5, 10 and 24 cm, while stocking the tank with a stepwise increasing amount of

seaweed (from 0 to 9.5 g FW L^{-1}). The pattern of exposure to light and darkness of an individual plant circulating inside the tanks was simulated by introducing a yellow neutrally buoyant plastic sponge as a thalli proxy. The period at the tank-surface and between individual surfacing events was measured 30 times.

Effects of cultivation conditions on photoinhibition

Photoinhibition was determined as the decrease of the potential quantum yield (F_v/F_m) of PS II (Hanelt 1996, Häder et al. 1998, Jiménez et al. 1998). Chlorophyll fluorescence emission was measured with a portable pulse amplitude modulated fluorometer (Diving-PAM, Walz, Effeltrich, Germany). Samples of *A. armata* (10 replicates) were placed in the fluorometer leaf-clip holders at a distance of 7 mm from the fibre optics and dark-adapted for 10 min. Subsequently, a saturating white light pulse (approx. 4000 µmol photons m^{-2} s^{-1}; 0.4s) was applied and F_v/F_m determined.

To assess the effects of different inoculation biomass densities on photoinhibition, tanks were incubated with the following biomass densities (n=2): 1.5 (in March), 4, 5, 6, 7, 8 and 9 g FW L^{-1} (in June). Water turnover rates within the tanks were adjusted accordingly to supply *A. armata* with non-limiting TAN fluxes (~100 and 200 µM h^{-1} in March and June respectively; see Schuenhoff et al. 2006 - Chapter 2). TAN flux was calculated as the product of the water turnover rate within the tanks and the average TAN concentration of the fish pond effluent along the day. The effects of different TAN fluxes on photoinhibition were assessed in January using a biomass density of 5 g FW L^{-1}. Mean daily TAN fluxes of 6, 17 and 34 µM h^{-1} (n=2) were adjusted as at this time of the year, this range of values include limiting and non-limiting TAN fluxes (Schuenhoff et al. 2006 - Chapter 2). All

culture conditions were maintained during a week before measurements. F_v/F_m was then measured along one day in 10 thalli randomly collected from each experimental tank.

The effects of an exposure to irradiance levels higher than photosynthetic saturation were determined by exposing *A. armata* to full solar irradiation (over 1600 µmol m^{-2} s^{-1}) for two periods of 1 and 3 hours. F_v/F_m was measured before full light exposure, every 30 minutes during light exposure and during a subsequent 2 hour period of recovery in the shade (50 µmol m^{-2} s^{-1}). Thalli exposed for 3 hours did not recover after 2 hours in the shade and were measured again after 17 hours.

Temperature effects on photosynthesis

Plants were collected from the tanks and immediately transported to the laboratory in a dark and cool container. Upon arrival, they were acclimated for 3 days in a growth chamber (Fitoclima 750 E, Aralab, Lisboa, Portugal) inside 250 ml glass flasks with GF/F filtered seawater under continuous aeration, at 15, 19, 24 and 29 °C. These temperatures cover the annual range found in the fish farm effluent. The growth chamber was set at a photoperiod of 14:10 (day:night) and a light intensity of 75 µmol photons m^{-2} s^{-1} (white light, Osram Lumilux Plus L18W/21-840). To test for acclimation effects, photosynthesis measurements were also made in plants obtained directly from the farm, which were exposed at a daily mean temperature of 25 ºC.

Photosynthesis was measured with a Clark type oxygen electrode (DW3 measuring chamber, Hansatech Instruments, Norfolk, UK). Samples of 3-6 mg DW were incubated in 15 ml GF/F filtered seawater while temperatures were maintained by a recirculating water bath (RayPa, Spain). Light was supplied by a slide projector (150 W halogen light bulb). Neutral density filters were used to obtain different irradiance levels. Net photosynthesis was

measured as the oxygen production (mg O_2 g $DW^{-1}h^{-1}$) at increasing irradiance levels (6.5 to 700 µmol photons m^{-2} s^{-1}). Respiration (R_d) was measured as the consumption of oxygen in the dark before the sequence of irradiances.

The Platt *et al*. (1980) model was selected to analyse the photosynthesis versus irradiance (P/I) data, because it contains a parameter of photoinhibition (β) and was the model that best fitted the observations:

$$P = P_s [1 – \exp (-\alpha I / P_s)] \exp (-\beta I / P_s)$$

where P stands for gross photosynthetic rate (mg O_2 g DW^{-1} h^{-1}), Ps for maximum photosynthetic rate (mg O_2 gDW^{-1} h^{-1}), I for irradiance (µmol photon m^{-2} s^{-1}), α for the ascending slope at limiting irradiance(mg O_2 g DW^{-1} h^{-1} (µmol photon m^{-2} s^{-1}) $^{-1}$ and β for photosynthetic decline at saturating irradiance. The SigmaPlot software package was used to fit the curves.

Statistical analysis

One way ANOVAs were performed to test for significant differences in the P/I photosynthetic parameters measured at different temperatures and to test for the effects of culture conditions on photoinhibition. When significant differences were found ($p \leq 0.05$), a Tukey HSD test was applied to test for significant differences in factor levels ($p \leq 0.05$)

Results

Cultivation conditions

The individuals of the *Falkenbergia*- phase of *A. Armata* consist of a "pompom" of intermingled filaments. Their pattern of circulation within the tanks, characterized by a short duration at the surface (about 1 second) and a longer period below it (about 9 seconds) result in an alternate pattern of light/dark exposure. Light availability within the tanks rapidly decreases with depth and with biomass density (Fig. 1). When inoculated at 2 g FW L^{-1}, more than 30% of surface PAR was available at a depth of 5 cm while below 24 cm, plants were in the dark. At 5 g FW L^{-1} only about 12% of the surface PAR was available at a depth of 5 cm and below 10 cm plants were already in the dark.

Effects of cultivation conditions on photoinhibition

Maximum values of potential quantum yield (F_v/F_m) were observed both in the morning and evening, whereas the minimum values occurred between 11:00 and 14:00, when irradiance was highest (Fig. 2a and 2b). With increasing density, the midday decline became less significant (Fig. 2b). F_v/F_m values were similar under all cultivation densities except at 1.5 and 4 g FW L^{-1} when they were significantly lower than the others, due to a significant midday decline. The effects of TAN flux on photoinhibition were only significant at a mean flux of 6 µM L^{-1} h^{-1} (Fig. 3). The maximum potential quantum yield of *A. armata* cultivated with this TAN flux experienced a significant midday decline but recovered to initial values later in the day.

Plants that were exposed to direct sunlight for one hour showed a significant decrease in F_v/F_m, from 0.61 ± 0.03 to 0.4 ± 0.03 (Fig. 4a). After a two hour period in the shade, F_v/F_m recovered to 86% of the initial quantum yield value. The longer exposure time of three hours led to a 39% decrease of the initial F_v/F_m values (Fig. 4b). In this case, F_v/F_m recovery was only up to 47% of the initial value. Even after a period of 17 hours in the shade, no further recovery was observed in these plants (data not shown).

Temperature effects on photosynthesis

The adjustment of the Platt et al. (1980) model to the P/I data was better (R^2=0.80) in the acclimated samples (Fig. 5; 15 ºC, 19 ºC, 24 ºC and 29 ºC), than in non-acclimated plants (Fig. 5; 25 ºC), where it only explained 59% of the data variance. A general decline in the photosynthetic rate (photoinhibition) of *Asparagopsis armata* with irradiances above 150 µmol m^{-2} s^{-1} was observed at all temperatures tested (Fig. 5). Maximum gross photosynthetic rates were similar for samples acclimated at temperatures between 15 and 24 ºC (~11 mg O_2 g DW^{-1} h^{-1}) while they increased dramatically to 46.6 mg O_2 g DW^{-1} h^{-1}at 29 ºC. This difference is explained by the plant's respiration that was lower than 5 mg O_2 g DW^{-1} h^{-1} in all samples, except at 29 ºC where there was a ten-fold increase (Fig. 6), suggesting the onset of a metabolic threshold. As well, the maximum net photosynthetic rates showed a slight but significant decrease with increasing incubation temperatures, from 9.74 ± 0.6 mg O_2 g DW^{-1} h^{-1} at 15 ºC to 6.63 ± 0.3 mg O_2 g DW^{-1} h^{-1} at 24 ºC, decreasing sharply to 0.43 ± 0.9 mg O_2 g DW^{-1} h^{-1} at 29 ºC (Fig. 6). The maximum net photosynthetic rates of non-acclimated farm samples, cultivated at a mean daily temperature of 25 ºC, were not significantly different from thalli maintained in the laboratory at 24 ºC (Fig. 6). On the other hand, dark respiration (R_d) of non-acclimated thalli was lower than that of acclimated thalli.

The initial slope of the curves (α) was similar in plants acclimated to temperatures between 15 and 24 ºC (~0.3 mg O_2 g DW^{-1} h^{-1} (µmol photon m^{-2} s^{-1}) $^{-1}$) and higher than non-acclimated plants (0.08 mg O_2 g DW^{-1} h^{-1} (µmol photon m^{-2} s^{-1}) $^{-1}$). At 29ºC, the slope increased dramatically to 6.9 mg O_2 g DW^{-1} h^{-1} (µmol photon m^{-2} s^{-1}) $^{-1}$.

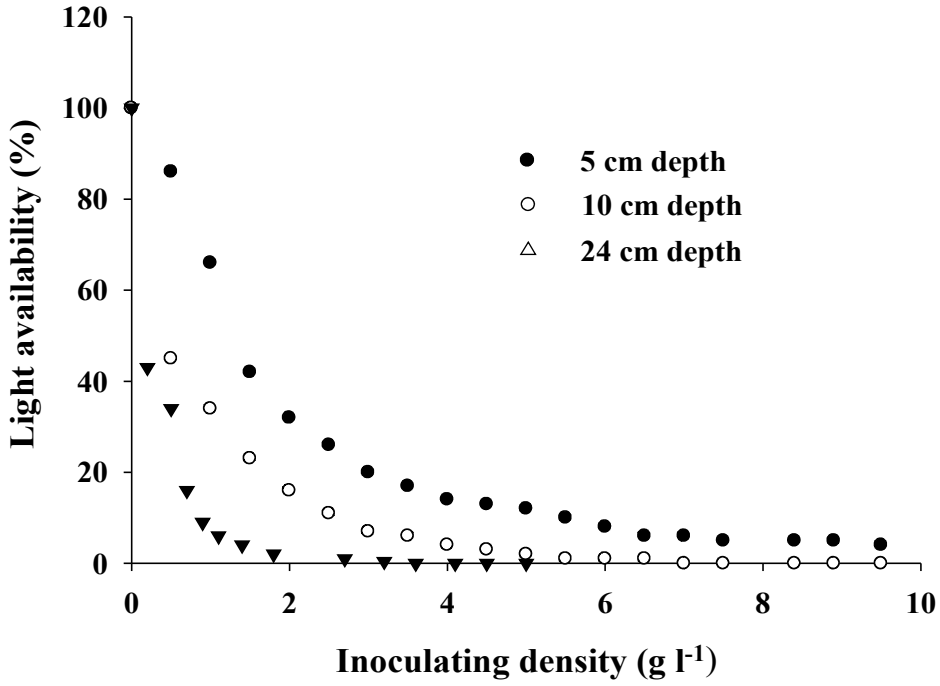

Fig. 1. Light availability at different biomass densities (g FW L^{-1}). Curves show measurements at 5, 10 and 24 cm depth within the tanks.

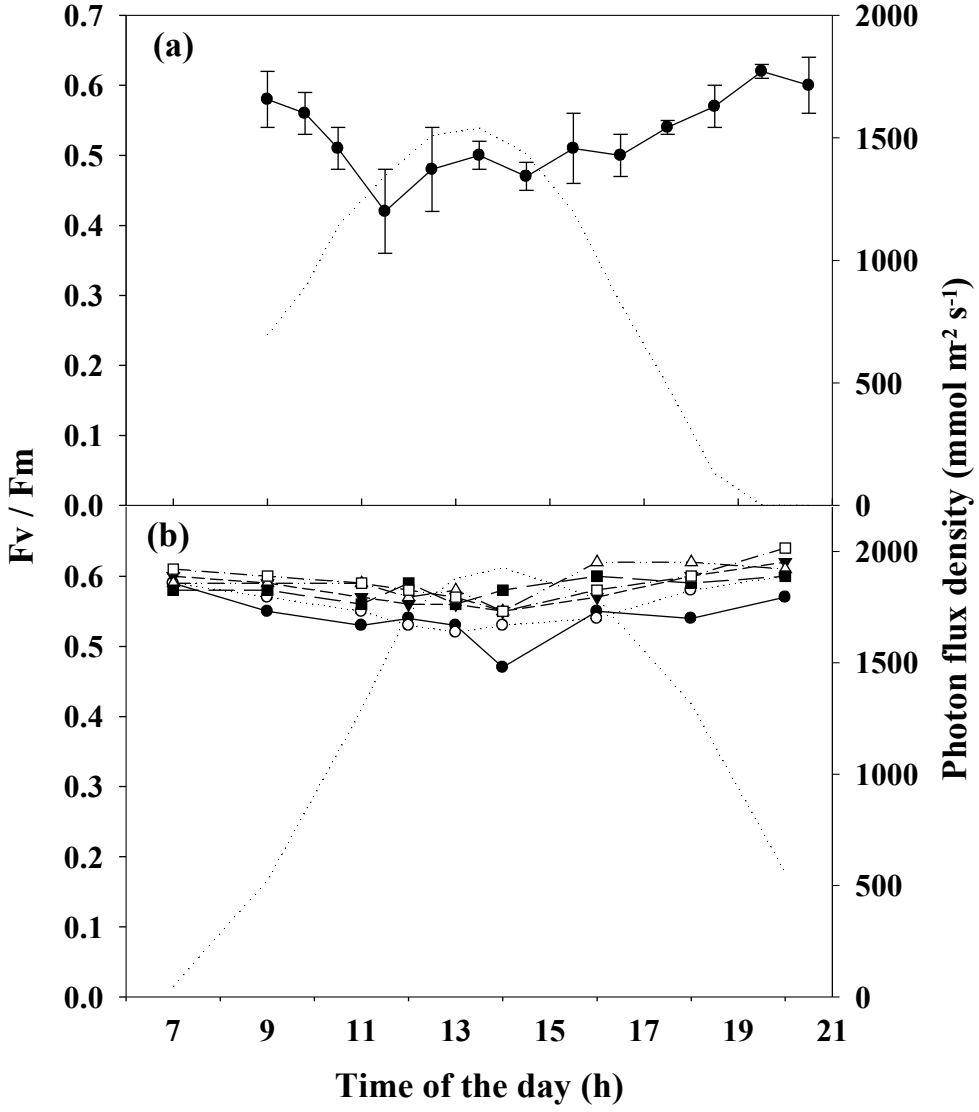

Fig. 2. Daily variation of *Asparagopsis armata* potential quantum yield (F_v/F_m) at different cultivation densities (a) Biomass density of 1.5 g FW L^{-1}, data collected in March; (b) Biomass densities of 4 (-●-), 5 (-○-), 6 (-▼-), 7 (-▽-), 8 (-▪-) and 9 g FW L^{-1} (-□-), data collected in June. Each data point is the average of 10 measurements. Standard deviations are only presented in graph (a). Dotted lines show the daily evolution of solar irradiance (µmol photon m^{-2} s^{-1}).

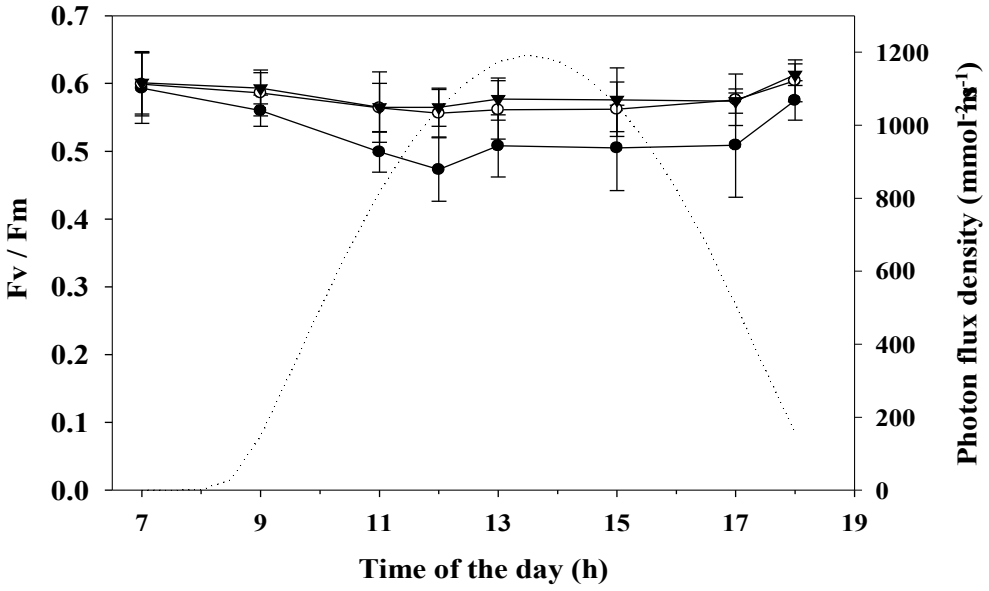

Fig. 3. Daily variation of *Asparagopsis armata* potential quantum yield (F_v/F_m) at different TAN fluxes: 6 (–●–), 17 (···○···) and 34 (–▼–) µM h^{-1}. Each data point is the average of 10 measurements. Bars show the standard deviations. Dotted line shows the daily evolutions of irradiance (µmol photon m^{-2} s^{-1}).

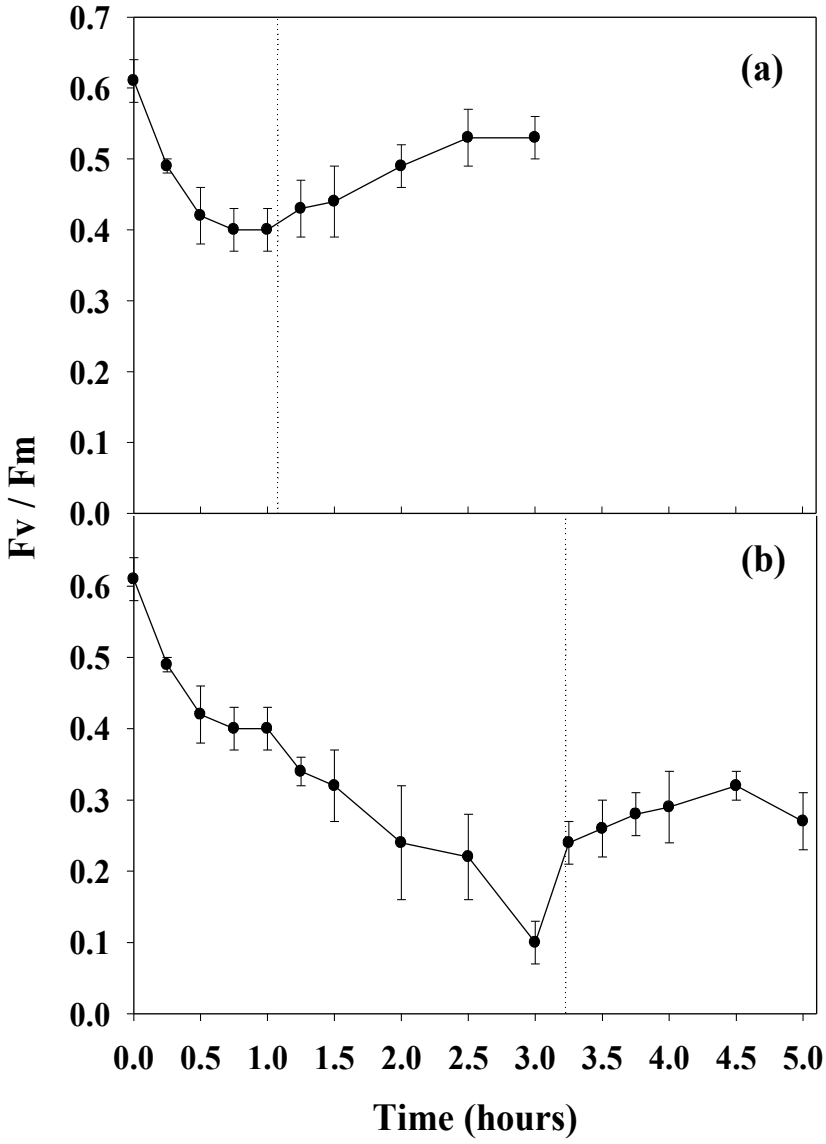

Fig. 4. Potential quantum yield (F_v/F_m) of *Asparagopsis armata* after exposure for 1 hour (a) and 3 hours (b) to direct sunlight (over 1600 µmol m^{-2} s^{-1}). The subsequent recovery in the shade (50 µmol m^{-2} s^{-1}) is represented on the right side of doted line.

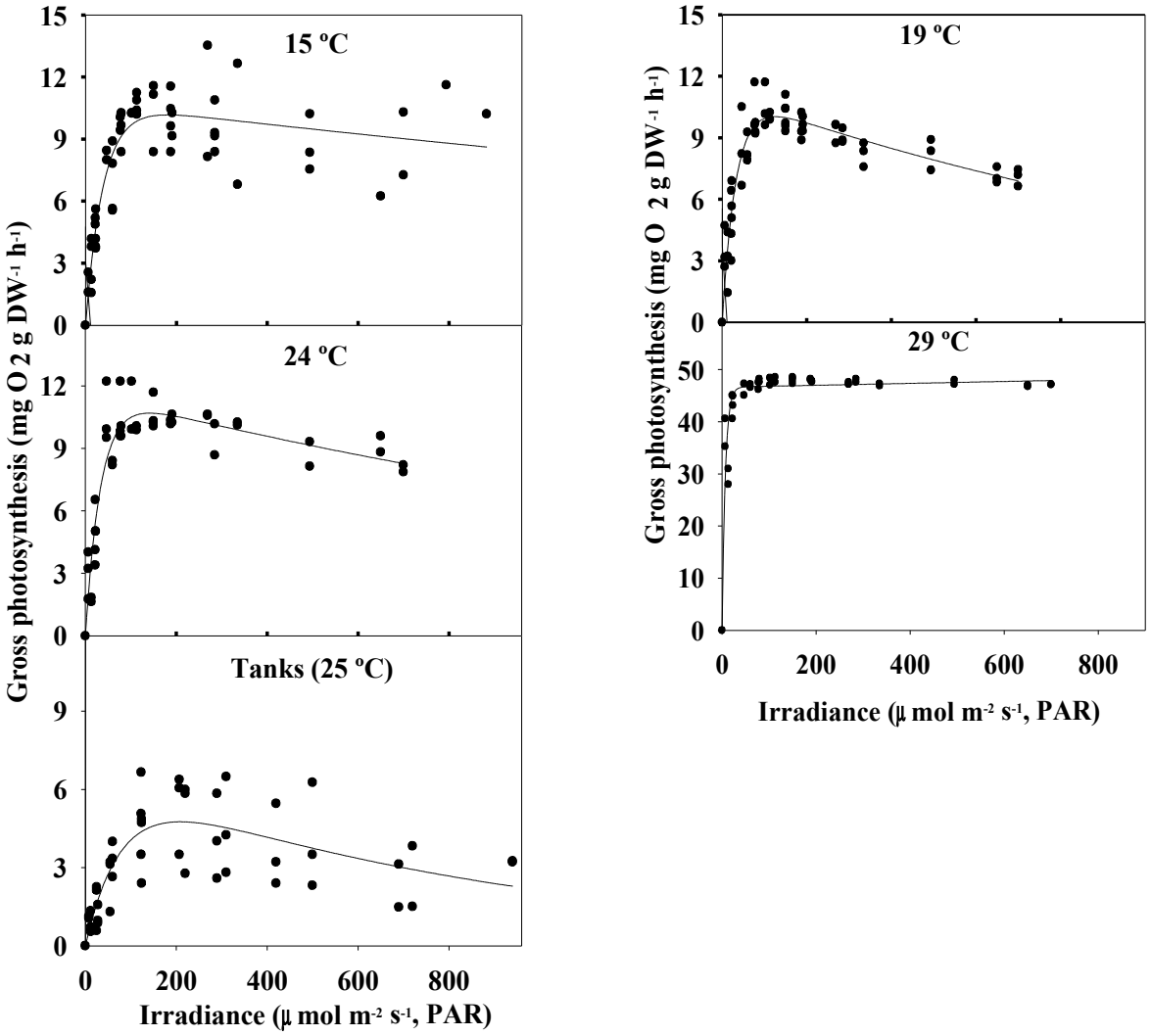

Fig. 5. Light response curves of non-acclimated (tanks) and acclimated *Asparagopsis armata* at 15, 19, 24 and 29 ºC. Curves were adjusted with the Platt et al. (1980) model.

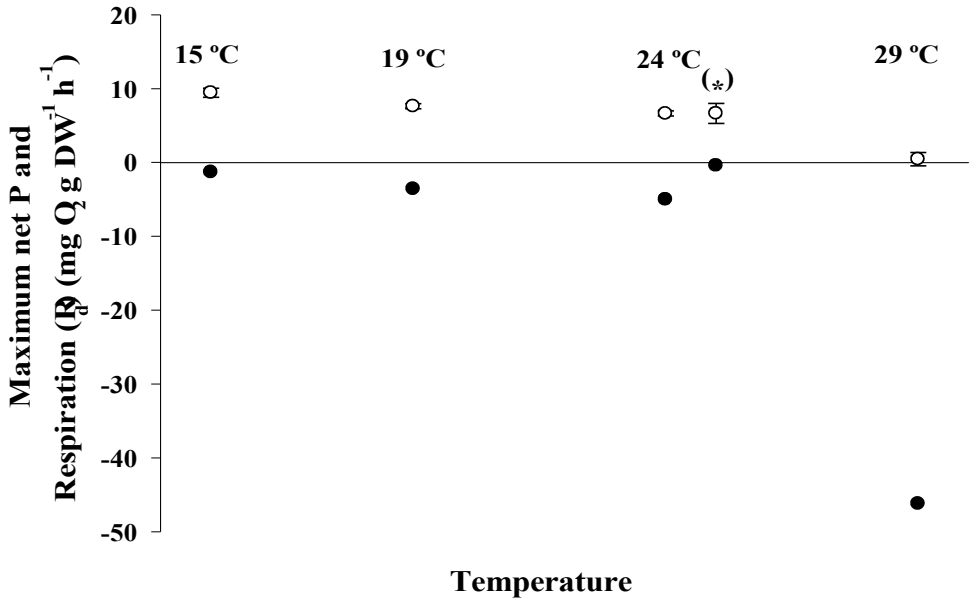

Fig. 6. Effects of temperature on the maximum net photosynthesis (○) and dark respiration (●) of both acclimated and non-acclimated *Asparagopsis armata*. Plants from the integrated aquaculture were at a temperature of 25 ºC (*). Values represent means±SE (n=6).

Discussion

Our results show that photoinhibition is not a major constrain for the integrated aquaculture of *Asparagopsis armata*. This is a protective mechanism for the photosynthetic apparatus to dissipate the excess of absorbed energy through fluorescence and heat, which has been widely described not only in natural stands of seaweeds (Ramus and Rosenberg 1980, Hanelt et al. 1993, Häder et al. 1996 a, b, 1998, Jiménez et al. 1998) but also in both seaweed (Aguirre-von Wobeser et al. 2000, Cabello-Pasini et al. 2000) and microalgae cultivation (e.g. Vonshak 2001). In the cultivation system presented here, the midday decrease of the photochemical efficiency (Fv/Fm) of *A. armata* was only observed at inoculation densities of 1.5 g FW L^{-1}, when the illuminated zone within the tanks was up to 24 cm deep. When cultivated at high densities, the compacted filamentous "pom-pons" of *A. armata* prevented light from penetrating below the first few centimetres of the surface zone. Plants spent around 10% of the time in this zone, where light levels may cause photoinhibition, but they probably had time to recover completely during the subsequent dark period. As outlined by Aguirre-von-Wobeser et al. (2000), the pulse type dosage of high PAR caused by the circulation of individual plants within the tanks may reduce photoinhibition. Even though cultivated *A. armata* individuals are most of the time in the shade, they do not behave strictly as shade-adapted plants as defined by Jiménez et al. (1998), because they showed a low degree of photoinhibition and a fast recovery in the shade, even when exposed to full solar irradiance during one hour.

A midday decline of Fv/Fm was also observed in the TAN flux experiment, but only for the lowest flux tested (5.7 µM h^{-1}). Even though *A. armata* was still nitrogen limited at a TAN flux of 17 µM h^{-1} (Schuenhoff et al. 2006 - Chapter 2), Fv/Fm was already insensitive

to this flux. This could be an indicator that at the lowest flux tested plants were under nutrient-starved conditions. Parkhill et al. (2001), provided evidence that Fv/Fm is only a sensitive indicator of nutrient-starved unbalance conditions, but when plants are acclimated to nutrient limitation, the relationship between Fv/Fm and nutrient stress fails. This fast and easy measurement of fluorescence may thus be used as a convenient diagnostic tool to detect nutrient-starved unbalance conditions of the cultures. This may be relevant to prevent plant physiological damage when, for example *A. armata* is cultivated under very low TAN fluxes in order to increase the efficiency of TAN removal from fish farm effluents (Schuenhoff et al. 2006 - Chapter 2).

Maximum net photosynthetic rates of *A. armata* peaked at 15 ºC and remained high over a wide range of temperatures, from 15 to 24 ºC, a common behaviour of macroalgae (Oates and Murray 1983, Baghdadli et al. 1990, Madsen and Maberly 1990, Davison 1991). The photosynthetic data is consistent with the literature results that report the lethal temperature limits of *A. armata* from the warm-temperate Mediterranean-Atlantic to be from 5 to 27 ºC (Orfanidis 1991) and the optimal temperature for growth to be from 10 ºC to 21 ºC (Oza 1989, Orfanidis 1991).

The maximum net photosynthetic rates obtained for *A. armata* acclimated to temperatures from 15º C to 24º C, varied from 9.7 to 6.6 mg O_2 g DW^{-1} h^{-1}. These rates are higher than those determined for other potentially farmable red seaweeds, such as *Gracilaria* spp. (2.6 – 5.2 mg O_2 g DW^{-1} h^{-1} in Rivers and Peckol 1995, 0.77 mg O_2 g FW^{-1} h^{-1} in Lee et al. 1999) and *Hypnea musciformis* (3.1 mg O_2 g DW^{-1} h^{-1}, Rosenberg et al. 1995). The functional-form model proposed by Littler et al. (1983) in which seaweed biomass productivity is higher in sheet-like species, followed by filamentous forms and by coarsely-branched ones explains the observed differences. On the other hand, the filamentous *A. armata* showed higher net photosynthetic rates than the sheet-like species of *Porphyra* spp.

(1.92 – 7 mg O_2 g DW^{-1} h^{-1} in Zhang et al. 1997, 7.68 mg O_2 g DW^{-1} h^{-1} in Aguilera et al. 1997). This suggests that *A. armata* is a very productive species under saturating light conditions.

Within the tanks of the integrated aquaculture the photosynthetic performance of the circulating thalli depends mostly on the light-limited portion of the P/I curve, defined by its initial slope (α), as they are only briefly exposed to saturating levels of light, being most of the time under very low light levels. The photosynthetic O_2 evolution in the light limited zone (α) is not significantly influenced by temperature but is mainly controlled by the light reactions (Falkowski and Raven 1997). The value of α measured in the *A. armata* plants from the integrated aquaculture suggests adaptation to light as it was lower than in the plants acclimated to the lower levels of light of the culture chambers in the laboratory. This α sensitivity to light, coupled to the ability of efficient use of saturating light levels at the water surface without damaging the photosynthetic apparatus, indicates that *A. armata* has a considerable photosynthetic plasticity. When the thalli emerge to the light zone they are again ready to use light in an efficient manner. It has been reported that photosynthesis is more efficient per unit of light when exposed to short flashes of intense light than under continuous light (Bidwell et al. 1985)

Care must be taken when applying the laboratory observations of the temperature effects on photosynthesis to the integrated aquaculture conditions as the light and temperature regimes are very different. While in the laboratory the plants are exposed to continuous levels of light (photoperiod) and temperature, in the aquaculture they are exposed to varying levels along the day.

The dramatic increase of respiration observed in the laboratory acclimated plants from 24 to 29 ºC suggests that a metabolic threshold was attained, in agreement with the 27º C lethal limit observed by Orfanidis (1991) for this species. This is a strong indicator that *A.*

armata integrated aquaculture in southern Portugal in the summer, when daily maximum water temperatures within the tanks may reach 29° C, may be difficult, in spite of putative seasonal adaptations. In fact, *A. armata* cultures were invaded by other species during the hottest summer period, due to the sharp decrease of net photosynthesis and consequently of growth rate. During the rest of the year, the species grew well and production peaked in spring with higher irradiances and photoperiod (Schuenhoff et al. 2006 - Chapter 2).

Our findings confirm *Asparagopsis armata* tetrasporophyte as a good candidate for commercial tank cultivation at temperatures up to 24º C. The species showed a high photosynthetic performance under a wide range of temperatures and irradiances. When cultivated at a biomass density of at least 5 g FW L^{-1}, there was no decrease in the photosynthetic performance due to photoinhibition.

CHAPTER 4

A direct comparison of two seaweed biofilters: *Asparagopsis armata* and *Ulva rigida*

Abstract

The tetrasporophyte of *Asparagopsis armata* was previously established as a novel seaweed biofilter for integrated land-based mariculture. The species growth and biofiltration rates were much higher than the values described in the literature for *Ulva* spp., the most common seaweed biofilter. However, to confirm this, the comparison should be performed at the same time under identical culture conditions. In this work we compared directly and in parallel the biofiltration performance and biomass yield of *A. armata* and *Ulva rigida*, cultivated in the effluents of a fish farm in southern Portugal. The effects of different water renewal rates on both the biomass yield and the TAN removal was tested for each species in winter and late spring.

The maximum TAN removal rates were similar for both species in December (2.7 and 2.8 g TAN m^{-2} d^{-1} for *U. rigida* and *A. armata* respectively), and higher for *A. armata* (6.5 g TAN m^{-2} d^{-1}) than for *U. rigida* (5.1 g TAN m^{-2} d^{-1}) in May. Higher differences were observed when estimating the nitrogen biofiltration through the organic nitrogen yield (N-yield) of the biomass produced, particularly in May. This estimate is directly related with the

biomass yield and the N content in the tissue, which were always higher for *A. armata* than for *U. rigida*. In December, the maximum biomass yields were 71g DW m^{-2} d^{-1} for *A. armata* and 44g DW m^{-2} d^{-1} for *U. rigida*, while in May the yield of *A. armata* was 125 g DW m^{-2} d^{-1} and of *U. rigida* was 73 g DW m^{-2} d^{-1}. To our best knowledge, these seaweed production rates are the highest ever reported for macroalgae cultivated in tanks.

Introduction

Research on seaweed biofilters for treating effluents from mariculture practices started in the mid-1970s (Ryther et al. 1975, Langton et al. 1977) and continued in the 80s with few isolated works (e.g. DeBusk et al. 1986, McDonald 1987). It was in the 1990s that this research field gained a renewed and increased interest (see the reviews by Chopin et al. 2001, Neori et al. 2004, Troell et al. 2003). Species from the genus *Ulva* were soon identified as ideal candidates for filtering fish effluents, due to their capacity to rapidly absorb and metabolize nitrogen, their high growth rates, their low epiphytism susceptibility and their world wide distribution (Jiménez del Rio et al. 1996, Neori et al. 2000, Msuya and Neori 2002, Mata and Santos 2003, Schuenhoff et al. 2003). However, seaweed biofiltration of fish farm effluents was not widely adopted by the aquaculture industry. The future development of this technology may probably depend on the progress of new regulations that enforce the fish farm companies to internalize the environmental costs of their operations (polluter-paying principle) and/or on an increased added value of the produced seaweed. Research efforts should focus on the cultivation of economic valuable seaweeds so that nutrient biofiltration may be identified by the aquaculture industry as a self sustainable, environmental friendly, technology that produces profitable biomass using a free source of nutrients, including CO_2.

We have previously established the tetrasporophyte of *Asparagopsis armata* as a novel seaweed biofilter for integrated land-based mariculture, which shows exceptional high growth and biofiltration rates (Schuenhoff et al. 2006 - chapter 2). Furthermore, *A. armata* is a rich source of halogenated organic compounds (McConnell and Fenical 1977) with remarkable antibacterial and antifungal activity that is being used as natural preservatives in cosmetic formulations. This species also produces sulphated galactans with potential

therapeutic applications (Braun et al. 1983, Caporiccio et al. 1983) and new sources of anti-HIV compounds (Haslin et al. 2001). A comparison with literature data of *Ulva* spp. performance indicated that *A. armata* may be a better biofilter (Schuenhoff et al. 2006 -chapter 2). However, it is problematic to compare biomass yield and TAN removal values when different cultivation systems were used, because factors such as tank design, tank depth, available light (stocking density, tank transparency), nutrients and water turnover rates will have a major effect on those parameters. To assess the relative performance of *A. armata* and *U. rigida,* as biofilters of fish farm effluents, we tested, at the same time and under the same culture conditions, the effects of different water renewal rates on the biomass yield, N-content and TAN removal rate of both species.

Material and Methods

Experimentation took place in an integrated fish/seaweed cultivation system established at Aquamarim, a fish farm in southern Portugal, which was previously described in Schuenhoff et al. 2006 (chapter 2). In December 2004 and May 2005, 12 cylindrical white (transparency ~70%) polyethylene tanks (Allibert Buckhorn C1100; 110 L capacity, 0.23 m^2 surface area), were supplied with particle filtered (150 μm) fishpond effluent. These periods were selected to compare the species growth and biofiltration performance during the lowest and highest temperature and irradiance seasons.

Six tanks were stocked with each species at previously established biomass densities that optimize yield. *A. armata* was stocked at 5 g centrifuged fresh weight (FW) L^{-1} (Schuenhoff et al. 2006 - chapter 2) and *U. rigida* at 4 g FW L^{-1} (unpublished data). Each tank was subjected to a different nutrient flux, which covered a range from very low water

exchange rates to the highest that was possible in this system (between 0.1 and 4 vol h^{-1}). In order to prevent individual variations between tanks, the biomass of each species was stocked in one tank of 1.4m^3 before the experiment and was supplied with sufficiently high effluent exchange rates to avoid nutrient starvation.

The duration of each experiment was one week. During this week, the temperature and the pH of the fish effluent (after the particle filter) were monitored, and water samples for TAN analysis were taken from the inflow and the outflow of each individual seaweed tank. These samples were collected every two days and at three different hours of the day (6:00, 13:00 and 16:00/18:00) to assess daily variations in the performance of each species. For TAN concentration analysis, duplicate water samples were filtered (0.25 µm; CF Whatman) into acid washed vials and taken immediately to the laboratory for analysis on a loop-flow analyser (uMAC-1000 multiparametric, Systea, Anagni, Italy) using the standard procedure described in Grasshoff (1983). The inflow and outflow TAN fluxes (µmol L^{-1} h^{-1}) were calculated as the product between the TAN concentration (µmol L^{-1}) of the incoming and outgoing water, respectively, and the water renewal rates (number of volumes h^{-1}) of each individual tank. Biofiltration in each tank was calculated as the difference between incoming and outgoing TAN fluxes. Michaelis-Menten type curves were fitted to plots of TAN flux versus both TAN removal and biomass yield data using least square nonlinear regression. Maximum TAN removal (V_{max}) and half saturation constants (K_s) were estimated.

At the end of the experiments, the biomass yield in each tank was calculated from the equation Y (g DW m^{-2} wk^{-1}) = (N_t - N_0) / t / (DW / FW) /A, where N_t is the final fresh weight, N_0 the initial fresh weight, DW/FW the dry weight / centrifuged fresh weight ratio and A the area covered by the tank in m^2. Triplicate seaweed samples of each tank (10 g FW) were taken at the beginning and end of the experiments and were oven-dried (48 h; 60 °C) both for DW/FW ratio determination and for tissue N content analysis using an elemental

analyser (Flash EA 1112, ThermoFinnigan). The N-yield of the produced biomass was calculated by multiplying biomass yield by the N content of the seaweed tissue. The N-yield is a better estimation of biofiltration than the TAN removal derived from the water analysis data because it integrates removal over a week whereas TAN removal was only assessed 3 times a day every two days. TAN removal was estimated considering that the 6:00 samples were representative of the whole night hours (15 h in December and 10 hours in May). During the day it was calculated considering the values obtained at 13:00 and 16:00 in December and 13:00 and 18:00 in May, and the number of light hours (9 in December and 14 in May).

Water temperature and pH were monitored using a portable pH probe (YSI model 63, Yellowsprings, OH, USA). A Li-190SA Quantum Sensor connected to a Li-1000 Data Logger (both LI-COR Inc., Lincoln, NE, USA) monitored open-air photon flux density (PFD) during the whole experimental periods.

Results

The daily averages of photon flux density (PFD) and water temperature were constant throughout each the experimental week. The daily PFD average was four times higher in May than in December (850 and 210 µmol m^{-2} s^{-1}, respectively). Maximum light intensity values recorded at 13:00 were 860 µmol m^{-2} s^{-1} in December and 1940 µmol m^{-2} s^{-1} in May. The water temperature in the fishpond had absolute maxima/minima of 24/19 °C in May, with a weekly average of 21.5±1.3 °C. In December, the daily variation of fishpond temperature was less pronounced and the weekly average was 14.2±0.5 °C. The average pH

of the effluent, measured at the solar midday, was 7.34 ± 0.12 in December and 7.48 ± 0.03 in May.

The TAN concentration of the fish farm effluent was relatively constant along each experimental week and was about 2-fold higher in May (52.5±8.6 μmol L^{-1}) than in December (26.0±3.2 μmol L^{-1}). The range of water renewals tested resulted in a range of TAN fluxes that varied from 4 to 135 μmol L^{-1} h^{-1} in December and from 1 to 250 μmol L^{-1} h^{-1} in May. This range of TAN fluxes resulted in a Michaelis-Menten type of TAN removal response in both experiments (Figs. 1 and 2). In general, TAN removal (both V_{max} and K_s) was lowest at dawn, peaked at mid-day and decreased to relatively high levels in the late afternoon, except in the case of *A. armata* in May, which V_{max} peaked in the late afternoon (Fig. 2, 18:00). It is noteworthy that at dawn, when PAR within the tanks was zero, TAN removal could be observed for both species. Under these dark conditions the estimated V_{max} for *A. armata* was around 40% higher than *U. rigida* in both seasons (Figs 1 and 2).

The *A. armata* biofilter was generally more efficient than *U. rigida*, except in December at mid-day (13:00, Figs 1 and 2) when the *U. rigida* curve was above *A. armata*. The highest *U. rigida* TAN removal rate was recorded in May, at around 70 μmol L^{-1} h^{-1}, when TAN fluxes were higher than 175 μmol L^{-1} h^{-1}, whereas the maximum estimated removal rate (V_{max}) was 83.4 μmol L^{-1} h^{-1} (Fig. 2). In December, the highest *U. rigida* V_{max} was 59.8 μmol L^{-1} h^{-1} and the highest recorded removal rate was 40 μmol of TAN L^{-1} h^{-1} with TAN fluxes around 100 μmol L^{-1} h^{-1}. Later in the day, with lower light levels, the maximum recorded values of TAN removal in both experimental weeks were similar to the estimated V_{max} values (Figs. 1 and 2), suggesting non-limiting fluxes of TAN for TAN removal. Concerning *A. armata*, highest TAN removal values of around 80 μmol L^{-1} h^{-1}, were recorded at 18:00 in May under TAN fluxes higher than 150 μmol L^{-1} h^{-1}. These values were still below the estimated V_{max}, 110 μmol L^{-1} h^{-1} (Fig. 2). In December, maximum removal

rates of around 30 μmol L^{-1} h^{-1} (about the same value of V_{max}) were achieved with fluxes higher than 100 μmol L^{-1} h^{-1} (Fig. 1).

Both species showed an asymptotic increase of biomass yield with TAN flux (Fig. 3). In December, at the maximum TAN-fluxes tested (around 100 μmol L^{-1} h^{-1}), *A. armata* biomass yield was 496g DW m^{-2} wk^{-1} whereas *U. rigida* was 306g DW m^{-2} wk^{-1}. Both maximum yield values were near the estimated V_{max} of the curve (548 and 362g DW m^{-2} wk^{-1}, respectively). The N-content at the time of stocking was 4.98±0.05 % DW for *U. rigida* and 5.90±0.05 % DW for *A. armata*. At the time of harvesting, each species showed a slight increase of their N-content, with an average of 5.25±0.18 % DW for *U. rigida* and 6.04±0.07 % DW for *A. armata*.

In May, the biomass yield of both species was significantly higher than it was in December. At maximum TAN-fluxes of around 200 μmol L^{-1} h^{-1}, the biomass yield of *A. armata* was 874 g DW m^{-2} wk^{-1} whereas of *U. rigida* yield was saturated (~500 g DW m^{-2} wk^{-1}) at much lower TAN fluxes (75 μmol TAN L^{-1} h^{-1}; Fig. 3). The N content of both species was lower in May than it was in December. When *A. armata* was stocked it had an N-content of 5.56±0.13 % and at the time of harvest it had 5.62±0.19 %. Stocked *U. rigida* had an N-content of 4.17±0.03 %, which increased at the end of the experiment to 4.8±0.19 %, except at the lowest TAN flux conditions where it decreased to 3.28±0.01 %.

A direct comparison of weekly biofiltration rates of *Ulva rigida* and *Asparagopsis armata* estimated by both TAN removal and N-yield approaches is presented in Figure 4. In general, the biofiltation estimated by the N_yield integrative approach is higher than the TAN removal approach, except for *U. rigida* in May. A comparison of maximum biofiltration rates of *U. rigida* and *A. armata* estimated here by both TAN removal and N-yield approaches is presented in the below section of Table 1. In December, when mean water temperatures were 14.5º C and TAN fluxes were 100 μmol L^{-1} h^{-1} similar TAN

removal rates were obtained. However, the biofiltration estimates through the N-yield approach show that the biofiltration of *A. armata* was 55% higher than *U. rigida*. In May, whereas the *A. armata* TAN biofiltration was 27% higher than *U. rigida* the N-yield biofiltration was more than 2-fold higher (Table 1).

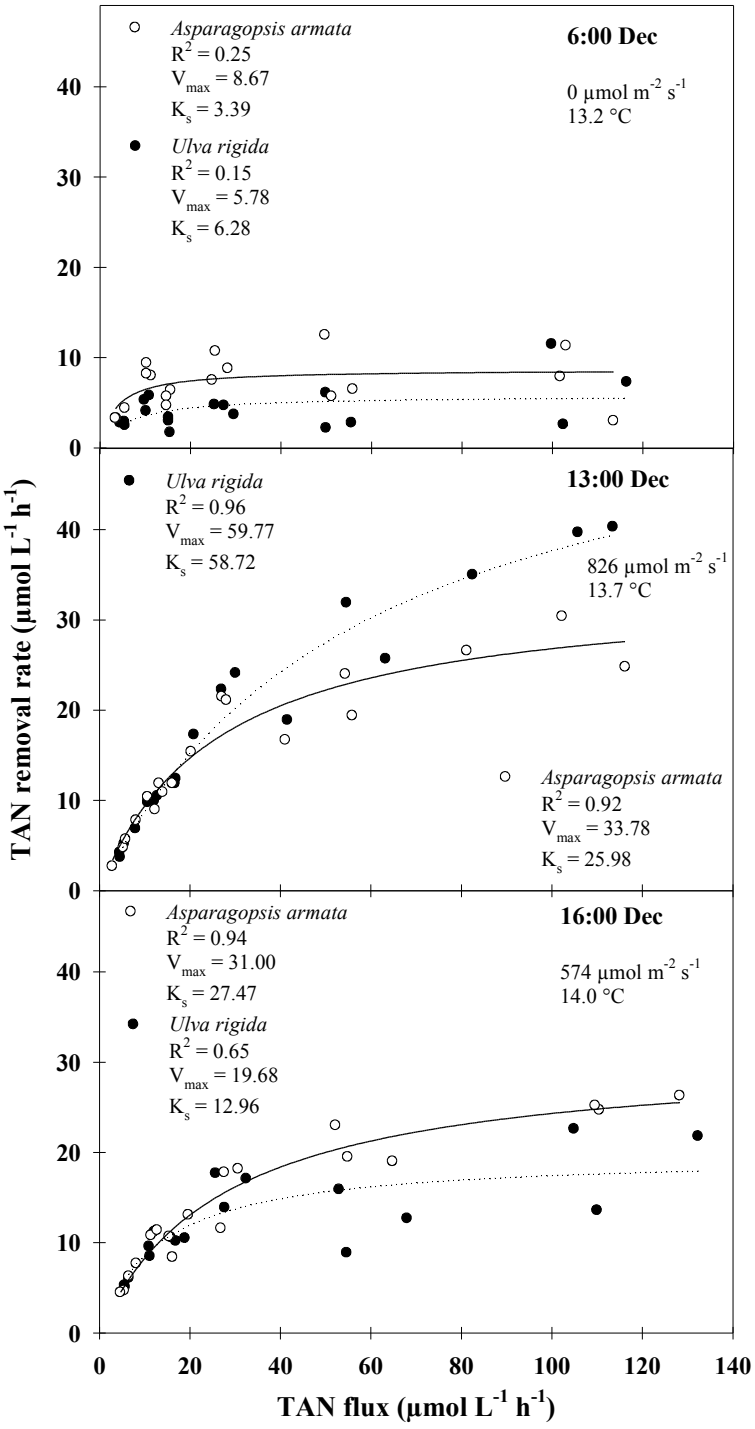

Fig. 1. Effects of TAN fluxes on TAN removal rates of *A. armata* and *U. rigida* in Winter. The coefficient of determination of curves (R^2) the maximum TAN removal (V_{max}) and the half saturation constants (K_s) are presented as well as the culture conditions (irradiance and temperature).

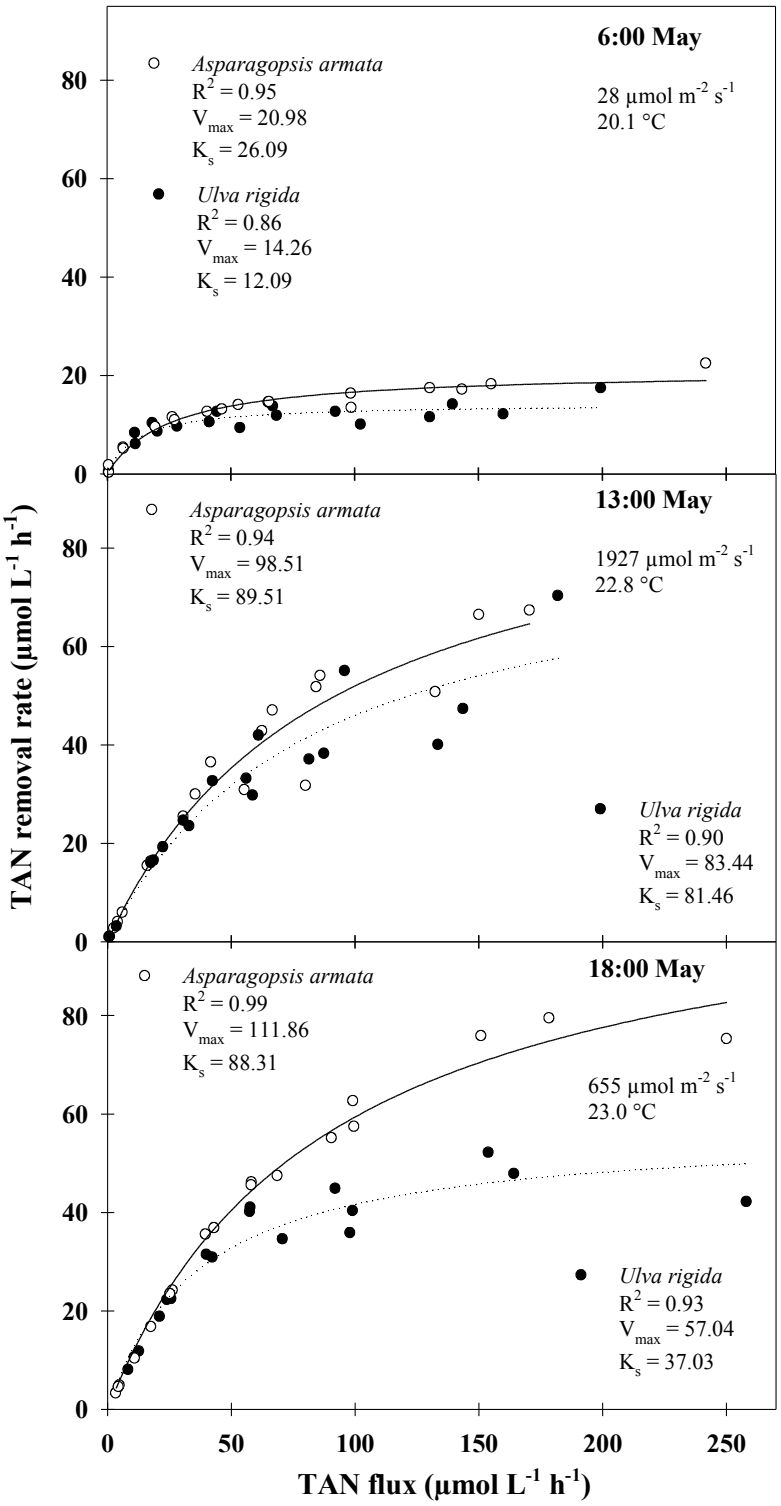

Fig. 2. Effects of TAN fluxes on TAN removal rates of *A. armata* and *U. rigida* in Spring. The coefficient of determination of curves (R^2) the maximum TAN removal (V_{max}) and the half saturation constants (K_s) are presented as well as the culture conditions (irradiance and temperature).

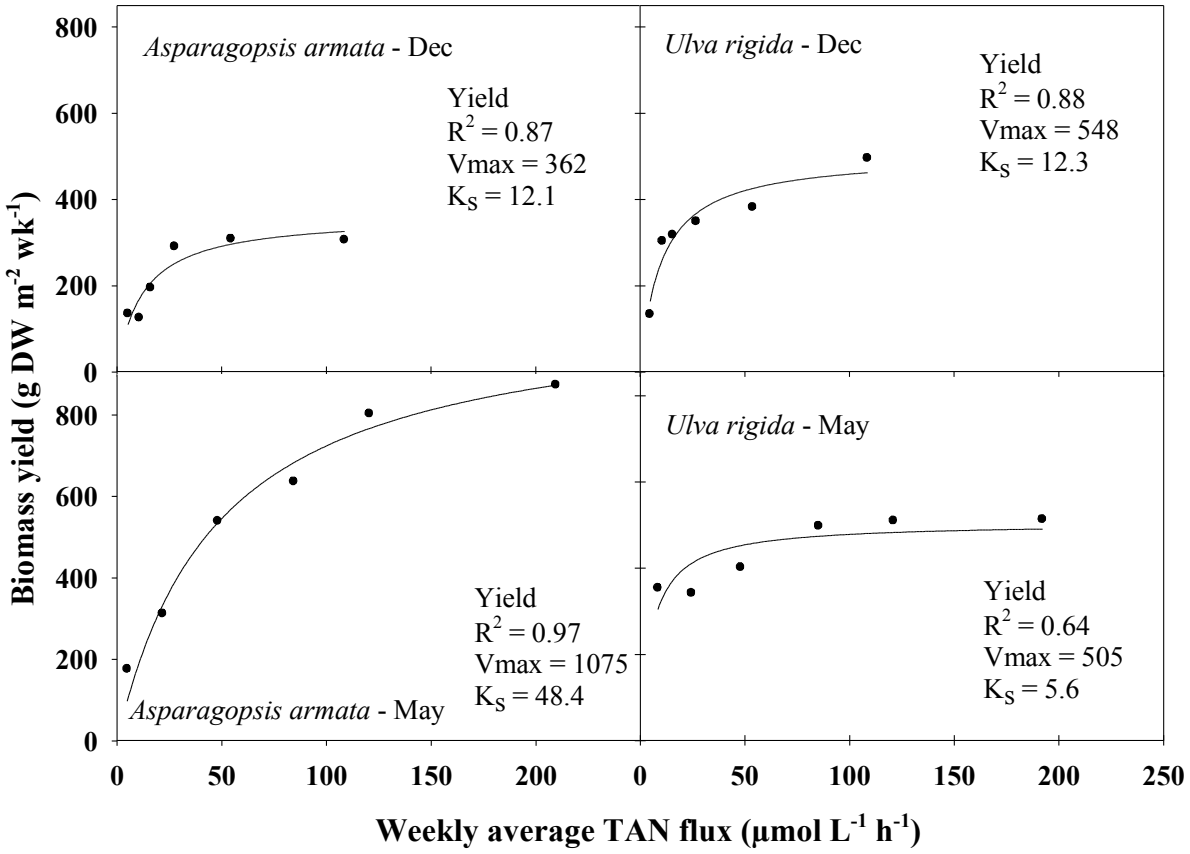

Fig. 3. Effects of TAN flux (weekly average) on the biomass yield of *A. armata* and *U. rigida*. The coefficient of determination of curves (R^2) the maximum biomass yield (V_{max}) and the half saturation constants (K_s) are presented.

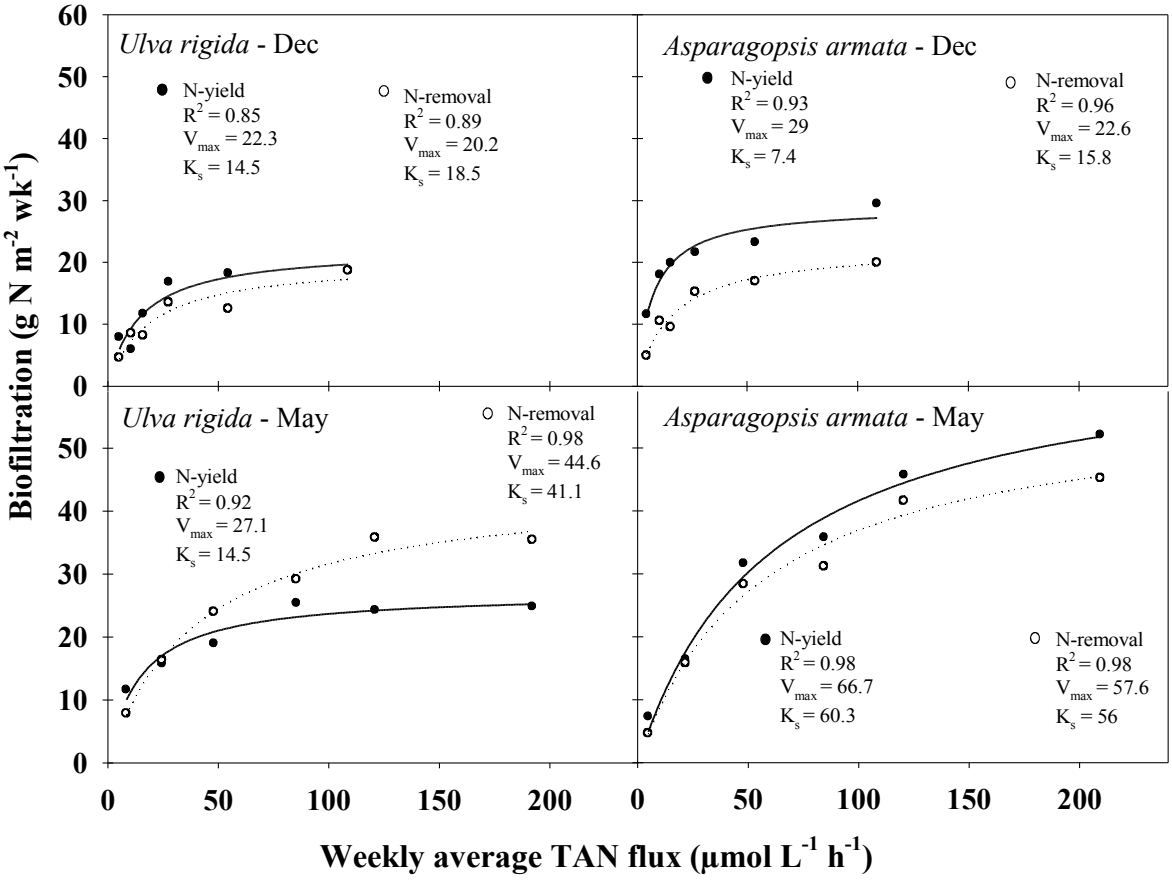

Fig. 4. Effects of TAN flux (weekly average) on biofiltration rates, estimated both by TAN removal and N-yield (see text). The coefficient of determination of curves (R^2) the maximum biofiltration rates and the maximum N-yield (V_{max}) and the half saturation constants (K_s) are presented.

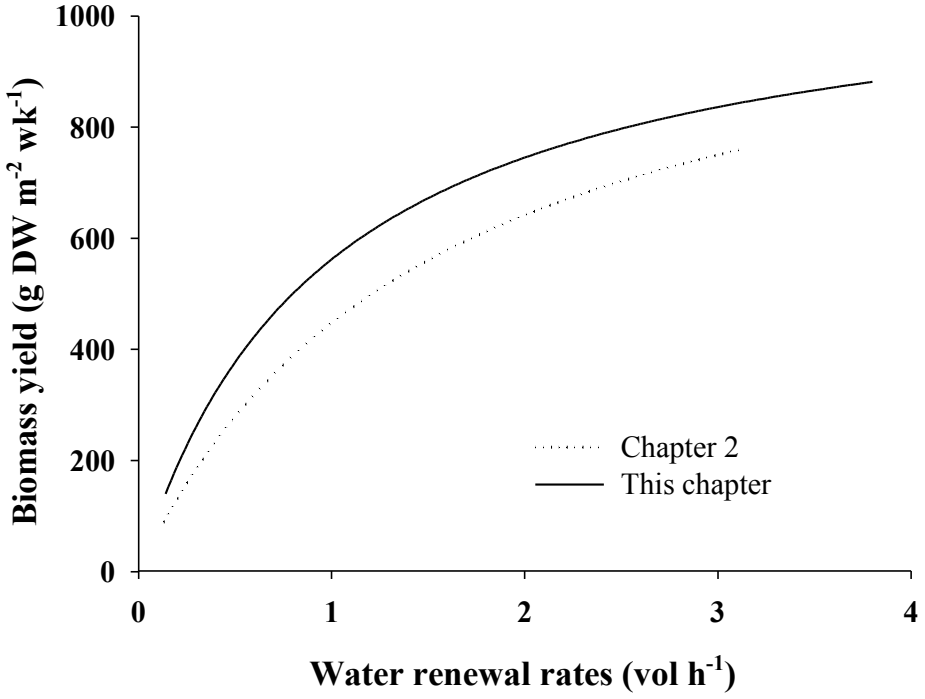

Fig. 5. Effects of water renewal rates on the biomass yield of *A. armata* in May: a comparison with Schuenhoff et al. 2006 (chapter 2) results.

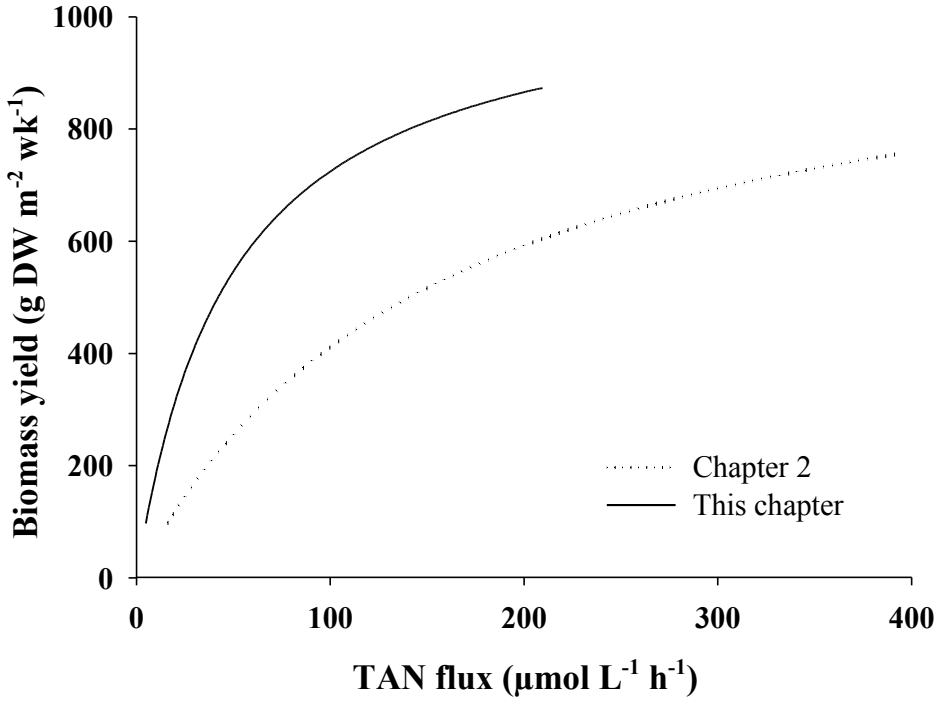

Fig. 6. Effects of TAN fluxes on the biomass yield of *A. armata* in May: a comparison with Schuenhoff et al. 2006 (chapter 2) results.

Table 1. Cultivation conditions, biofiltration and biomass yields of *Ulva* spp. and *Asparagopsis armata*.

Species	Tank volume L	Stocking density g FW L^{-1}	Mean water temperature °C	Maximum Water exchange Vol h^{-1}	TAN flux µmol L^{-1} h^{-1}	TAN removal µmol L^{-1} h^{-1}	Daily TAN removal g m^{-2} d^{-1}	Biomass yield g DW m^{-2} d^{-1}	N-yield g m^{-2} d^{-1}	Reference
Ulva lactuca	600	1.7	20	0.5	40	23	3.2	55	2.3	Cohen and Neori 1991, Neori et al. 1991
Ulva rigida	750	2.5	24	0.5	46 [a]	18	2	40	1.8	Jiménez del Rio et al. 1994, 1996
Ulva rigida	1900	2	22	0.6	18	10	1.31	48	1.45	Mata and Santos 2003
A. armata	110	5	13	1.3	35	12	1.1	43	2.7	Chapter 2 (Schuenhoff et al. 2006)
A. armata	110	5	22	3	500	100	8.8	120	5.9	Chapter 2 (Schuenhoff et al. 2006)
A. armata	110	5	14.2	4.1	100	30	2.8	71	4.2	This chapter
U. rigida	110	4	14.2	4.1	100	40.3	2.7	44	2.7	This chapter
A. armata	110	5	21.5	3.8	175	79.4	6.5	125	7.4	This chapter
U. rigida	110	4	21.5	3.8	175	70.2	5.1	73	3.6	This chapter

[a] reported as the total of dissolved inorganic nitrogen which includes nitrite and nitrate

Discussion

The direct comparison between the performance of *Asparagopsis armata* and *Ulva rigida* as biofilters for integrated, inland, fish/seaweed aquaculture, confirmed the hypothesis rose in Schuenhoff et al. 2006 (chapter 2) that *A. armata* is the most efficient biofilter. The present experiments, performed at the same time and under the same culture conditions, revealed that the differences are lower than what might be concluded by comparing the values reported in the literature (Table 1).

The higher performance of a filamentous form species over a sheet-like species would not be expected according to the functional-form model proposed by Littler et al. (1983). However, the specific light regime in the tanks may present different advantages to the different morphologies. The round-shape, "pom-pom" type morphology of *Asparagopsis* spp. tetrasporophytes rollover constantly in the aerated cultures, which increases the light/dark frequency conditions to the cells, in comparison with the blade type morphology of *Ulva* spp. Grobbelaar *et al.* (1996) revealed that seaweed productivity, and in particular that of low light acclimated algae, can be enhanced between 1.68 and 6 times with this type of light regime. *Asparagopsis* spp. in the dense culture tanks are low light acclimated (Mata et al. 2006 - Chapter 3).

Biofiltration and biomass yields per surface area obtained for *Ulva rigida* in this cultivation system were the highest ever reported for *Ulva* spp. cultivated in integrated fish/seaweed aquaculture (1.6-fold and 1.3-fold higher, respectively, Table 1). The remarkable performances obtained for both genera in this system may be probably related with the characteristics of the cultivation system itself. These tanks have a smaller volume (Table 1) and the wall is translucent, allowing the penetration of about 70% of the incident

PFD, which increases the light exposure surface / culture volume compared with tanks used elsewhere. This higher light availability to the plants per tank volume and per surface area, resulted in an ideal stocking biomass density around 2-fold higher than the biomass stocking densities used in other studies (Table 1). Such higher amount of biomass per square meter resulted in higher nutrient uptake rates and biomass yield per surface area of the cultivation system.

Besides light, the seaweed performance may also be limited by the availability of nutrients. The kinetics of nitrogen uptake is usually interpreted in terms of the Michaelis-Menten model, increasing with its availability in the medium until saturation (D'Elia and DeBoer 1978, Haines and Wheeler 1978). To maximize *U. rigida's* biofiltration capacity in this cultivation system at full light midday it was necessary to supply the tanks with TAN fluxes higher than 120 and 200 μmol L^{-1} h^{-1} in winter and late spring, respectively. Even though this biofiltration response model to TAN fluxes may not be directly applied to other cultivation systems, it suggests that the much lower TAN fluxes supplied elsewhere to the *Ulva* spp. cultures (Table 1) were limiting and explain why in most of them the plateau of the TAN uptake curve was not reached. Most of the *Ulva* spp. cultivated in integrated aquaculture has been done under the nutrient saturation level. Regarding *A. armata*, the biofiltration response to TAN fluxes obtained here confirmed what we had previously established (Schuenhoff et al. 2006 - chapter 2). In late spring TAN fluxes of at least 200 μmol L^{-1} h^{-1} saturates the *A. armata* TAN removal (~100 μmol L^{-1} h^{-1}), whereas in winter maximum TAN removal rates of 30 μmol L^{-1} h^{-1} can be achieved if the plants are supplied with at least 120 μmol L^{-1} h^{-1}.

The two approaches for estimating daily biofiltration, based on the TAN removal and on the N-yield, do not show a consistent pattern, the TAN removal estimates may be higher or lower than the N-yield estimates (Table 1). Considering only the results of the present work,

the TAN removal estimates are lower than the N-yield estimates for *A. armata*, whereas the opposite was observed for *U. rigida* in May, when TAN estimated biofiltration was 5.1g m^{-2} day^{-1}, a value 1.4-fold higher than the N-yield estimate. This difference results from a low *U. rigida* biomass yield rather than low N content, which was relatively stable. This was the result of the observed, but not quantified, mechanical breakage of the thalli and washout of the small fragments through the outflow, particularly in the tanks with high water renewal. This is a common occurrence in *Ulva* spp. cultivation (see e.g. Neori et al. 1991) that is often related to the sporulation of blade margins and consequent drop in biomass, highlighting the importance of using both estimates to assess *Ulva* biofiltration.

The *A. armata* yield data obtained in May at different water turnover rates is very similar with the data reported in Schuenhoff et al. 2006 (chapter 2) at identical water renewal values (Fig. 5). However, converting the water renewal rates to TAN fluxes in both studies, the patterns varied markedly (Fig. 6). The maximum biomass yield values obtained in this study were achieved with much lower TAN fluxes than in Schuenhoff et al. 2006 (chapter 2). Under identical TAN fluxes the biomass yield values were higher than those reported previously. This is an indication that other elements besides TAN influence the production of *A. armata*. Fish farm effluents are also naturally rich in dissolved CO_2 due to the respiration activity of both fish and micro-organisms within the ponds. It is known that algae cultivated at high biomass densities quickly deplete the DIC pool in the water becoming limited (Bidwell et al. 1985). What it is not known so far is if the extra CO_2 in the fishpond effluents is enough to maximize the biomass seaweed production in integrated aquaculture and how susceptible is the *A. armata* production to the available dissolved inorganic carbon quantity and forms. This issue is addressed in Chapter 5.

This study confirmed that *A. armata* is indeed a more efficient biofilter than *Ulva rigida.* Both species have all the potential to be intensively cultivated in tanks using

mariculture effluents as free sources of nutrients and carbon to maximize their production. In fact, the cultivation of both species in the same integrated cultivation system may make sense, especially in southern Portugal, where the *A. armata* production crashes during summer due to high temperatures (Schuenhoff et al. 2006 - chapter 2, Mata et al. 2006 - chapter 3). This approach of product diversification will also benefit the farmer. *Ulva* biomass is still sub-valorised, but is now gaining interest as a potential source of cell wall polysaccharides (especially ulvan), which physicochemical and biological properties make them attractive candidates for novel functional and biologically active polymers for the food/ feed, pharmaceutical, chemical aquaculture, and agriculture domains (Lahaye and Robic 2007).

CHAPTER 5

Is the tetrasporophyte of *Asparagopsis armata* (Bonnemaisoniales) limited by inorganic carbon in integrated aquaculture? *

Abstract

Seaweeds cultivated in traditional land-based tank systems usually grow under carbon-limited conditions and consequently have low production rates, if no costly artificial source of inorganic carbon is supplied. In integrated aquaculture, the fish effluents provide an extra source of dissolved inorganic carbon (DIC) to seaweeds due to fish respiration. To evaluate if the tetrasporophyte of *Asparagopsis armata* (Harv.) F. Schmitz (the *Falkenbergia* stage) is carbon limited when cultivated with effluents of a fish (*Sparus aurata*) farm in southern Portugal, we characterized the DIC forms in the water, assessed the species photosynthetic response to the different DIC concentrations and pH values, and inferred for the presence of a carbonic anhydrase (CA)–mediated mechanism. Results showed that *A. armata* relies mainly on CO_2 to meet photosynthetic needs. Nevertheless, from pH 7.5 upward, the CO_2 supply to RUBISCO seems to derive also from the external dehydration of HCO_3^- mediated by CA. The contribution of this mechanism was essential for *A. armata* to attain fully saturated O_2-evolution rates at the natural seawater DIC concentration (2–2.2 mM) and pH values (~8.0). We revealed in this study that seaweeds cultivated in fish-farm effluents

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benefit not only from a rich source of ammonia but also from an important and free source of DIC for their photosynthesis. If supplied at relatively high turnover rates (~100 vol d^{-1}), fish farm effluents provide enough carbon to maximize the photosynthesis and growth even for species with low affinity for HCO_3^-, avoiding the artificial and costly supply of inorganic carbon to seaweed cultures.

Introduction

Adequate carbon supply is an essential requirement for successful algal cultivation. When cultivated at high biomass densities a rapid depletion of the dissolved inorganic carbon (DIC) pool in the water occurs (Bidwell et al. 1985). Air bubbling supplies some CO_2 to the cultures, but the rate of carbon assimilation by the plants is much greater than the rate of CO_2 diffusion from the air into seawater even in open and vigorously aerated algae cultivation units. In general, the algae in cultivation suffer from carbon malnutrition, with consequent low rates of production (Bidwell et al. 1985, McLachlan et al. 1986, Jiménez et. al. 1995).

Carbon limitation can be avoided by supplying the cultures with CO_2 gas (Bidwell et al. 1985, Craigie and Shacklock 1989), by adding $NaHCO_3$ (Simpson et al. 1978) or CO_2 and $NaHCO_3$ (Demetropoulos and Langdon 2004) and by adding inorganic/organic acids (Debusk and Ryther 1984, Amat and Braud 1993). However, the use of these extra carbon sources represents a major operational cost; as much as 73% (Braud and Amat 1996). The development of less expensive methods of supplying DIC is one of the main issues to be solved in mass algae cultivation systems (Bidwell et al. 1985, Benemann et al. 1987, Oswald 1988, Tapie and Bernard 1988). The use of carbon enriched seawater sources was proposed by Braud and Amat (1996), who showed that mixing underground salt water (with a higher DIC concentration) with natural seawater, reduced the operational costs to maximize biomass yield. As well, the use of fish farm effluents for seaweed cultivation (integrated fish/seaweed aquaculture), provides the plants with a supplementary source of dissolved inorganic nutrients, including carbon. The benefits of the extra nitrogen and phosphorous sources from fish excretions for algae production are well described in the literature (reviewed by Neori et al. 2004). On the other hand, to the authors' knowledge, no research

has addressed if the extra DIC from fishpond effluents is enough to maximize the biomass production in integrated aquaculture.

The CO_2 derived from fish respiration dissolves in the culture medium and becomes part of a buffer system in which all DIC forms are in equilibrium. CO_2 is hydrated to produce carbonic acid and its subsequent deprotonation leads to the formation of bicarbonate and carbonate anions as expressed by the following equations: (1) $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$ (Falkowski and Raven 1997). The relative amount of these fractions in the DIC pool is pH dependent and, to a lesser extent, salinity and temperature dependent (Loban and Harrison 1997). In normal seawater (pH = 8 – 8.2), the DIC bulk is mainly composed by HCO_3^- (~95%), while the dissolved CO_2 fraction is less than 1%.

All seaweeds use CO_2, which is fixed by Rubisco in the chloroplasts (Falkowski and Raven 1997). While some species seem to be restricted to the passive diffusion of dissolved CO_2 for photosynthesis, others have developed mechanisms to use the most abundant DIC form in the medium (HCO_3^-) as an alternative source of carbon (Beer 1994, Larsson and Axelsson 1999). Several mechanisms of HCO_3^- utilization by seaweeds have been described (Maberly 1990, Raven 1991, 1997). The most common mechanism to all algae groups requires the periplasmic carbonic anhydrase (CA) to mediate dehydration of HCO_3^- into CO_2, which can be transported into the cell (Haglund et al. 1992a, b, Mercado et al. 1998, Larsson and Axelsson 1999).

The present study develops from ongoing research on the cultivation of the tetrasporophyte of *Asparagopsis armata* (the *Falkenbergia* stage), using fish farm effluents with the aim of removing the N load of this effluent and producing economically valuable biomass for the extraction of halogenated secondary metabolites (Schuenhoff et al. 2006 -chapter 2, Mata et al. 2006 - chapter 3). The objective of this work was to investigate if integrated fish/*A. armata* cultivation is carbon limited. To evaluate the importance of the

extra DIC source from fish effluents to *A. armata* production, we characterized the DIC forms availability in the water before and after passing the fish and seaweed units. We specifically assessed the relationship between *A. armata* photosynthesis and both DIC concentration and pH values, as well as the presence of a CA mediated mechanism and its operation conditions in *A. armata* under cultivation conditions.

Materials and methods

Cultivation parameters

The experiments were performed using an *Asparagopsis armata* cultivation unit in a land-based fish (*Sparus aurata*) farm in southern Portugal (for cultivation details see Schuenhoff et al. 2006 - chapter 2). In May 2005, six cylindrical white (transparency ~70%) polyethylene tanks (110 L capacity, 0.23 m^2 surface area; Allibert Buckhorn model C1100, Manterre, France), containing 5 g/L of biomass were kept at various, manually adjusted and carefully monitored, water flow rates ranging from a minimum of 0.1 vol h^{-1} to a maximum of about 4 vol h^{-1} during one week. This was the maximum possible flow of this cultivation system without overflowing. Salinity, temperature and pH were monitored in the water entering the fish unit, after passing the fish unit (i.e. before the seaweed tanks) and in the outflow water of all the 6 seaweed tanks at 06:00, 13:00 and 18:00 hours during three days. These parameters were measured using a pH probe (YSI model 63, Yellowsprings, Oiho, USA). At the same time, three water samples from each sampling site were collected to determine alkalinity. To avoid alkalinity changes until analysis, samples were stored in ~100

mL Winkler flasks with 200 µL of saturated $HgCl_2$ solution and were immediately taken to the laboratory at 4º C. Alkalinity was calculated from linear Gran-plots (Gran 1952) after potentiometric titration of 137 mL sample with 0.05 N HCl (Bradshaw et al. 1981). DIC concentration was calculated from the pH, alkalinity, salinity and temperature values of the original medium, using the software developed by Lewis and Wallace (1998). A Li-190SA Quantum Sensor connected to a Li-1000 Data Logger (both LI-COR Inc., Lincoln, NE, USA) monitored open-air photon flux density (PFD). Weekly mean photon flux during the day was 850 µmol m^{-2} s^{-1}.

The amount of the available DIC to the plants (DIC fluxes) was calculated as the product between the DIC concentration of the inflow water to the seaweed tanks (mmol L^{-1}) and the tank water renewal rate (L h^{-1}). The DIC fluxes leaving the tanks were calculated using the DIC concentration in the outflow of the seaweed tanks. The amount of DIC removed by the plants was calculated as the difference between the outflow and the inflow DIC fluxes. The DIC removal rates at 13:00 hours of the three sampling days were plotted against the DIC fluxes. A Michaelis-Menten curve was fitted to the plots to allow an estimation of maximum DIC removal (V_{max}) and half saturation constants (K_s). At the end of the experimental week, the biomass was weighted and weekly growth was calculated from the equation:

$$Y \text{ (g DW } m^{-2} \text{ } wk^{-1}) = (N_t - N_0) / (DW / FW) / A,$$

where N_t is the final fresh weight, N_0 the initial fresh weight, DW/FW the dry weight / centrifuged fresh weight ratio (~0.25) and A is the surface area of the tanks in m^2.

Photosynthetic oxygen evolution experiments.

Laboratory cultures of *Asparagopsis armata* provided the biomass for the photosynthesis experiments. These were being cultured at a temperature of 15º C and at an irradiance of 65 µmol m^{-2} s^{-1} during the 14 hours of the light period. O_2 evolution was measured with a Clark type oxygen electrode (DW3 measuring chamber, Hansatech Instruments, Norfolk, UK), at the optimal temperature (15º C) and saturating irradiance (160 µmol photons m^{-2} s^{-1}) as described in Mata et al. 2006 (chapter 3). To test the effects of pH on the photosynthetic O_2 evolution, algal samples with 10 to 20 mg of fresh weight were incubated in the chamber containing 15 mL of natural seawater buffered at the selected pH values. A known amount of biological solid buffer (Sigma) with a pK_a appropriate to the experimental pH was added to provide a final concentration of 25 mM, and the pH was then adjusted as desired with freshly prepared 1M NaOH and HCl solutions. The buffers used were Mes for pH 6.5, Hepes for pH 7 and 7.5, Tris for pH 8, 8.5 and 9 and Caps for pH 9.5 and 10.

O_2 evolution rates were also measured at different DIC concentrations at pH values of 6.5, 8, 8.5 and 9. These pH values were chosen as they are in the range of those measured in the cultivation tanks at the different water turnover rates (8 to 9). A pH of 6.5 was selected to study the photosynthetic performance of the plants in conditions where CO_2 is most probably not limiting to the plants. Photosynthetic rates were measured as previously described, but in this case using DIC-free artificial buffered seawater (450 mM NaCl, 30 mM $MgSO_4$, 10 mM KCl and 10mM $CaCl_2$; Beer et al. 1990). Samples were left to photosynthesize in this DIC-free artificial seawater for one hour before the start of the experiments to deplete all DIC eventually present in the cells. Desired DIC concentrations were added to the photosynthetic chamber in the form of HCO_3^-, from a 200 mM $NaHCO_3$ stock solution. When a constant O_2

evolution rate was reached, the carbonic anhydrase (CA) inhibitor acetazolamide (AZ) at a final concentration of 200 µM (Zou et al. 2004) was injected into the chamber and the O_2 evolution was again recorded. It is generally assumed that AZ cannot penetrate the plasmalemma and therefore inhibits only the extracellular CA activity (Haglund et al. 1992a). Medium and samples were replaced after each measurement. DIC maximum rate of O_2 evolution (V_{max}) and the concentration of DIC ($K_{0.5}$) supporting half of the V_{max} were estimated using the double reciprocal plot (Lineweaver-Burk).

Prior to all experiments the net photosynthetic rates of *A. armata* were determined at pH 6.5, 7.5, 8.5 and 9.5 with and without the addition of 25 mM of Mes, Hepes, Tris and Caps buffer respectively, to test for possible inhibitory effects of the buffer on photosynthesis. Inhibitory effect of the Tris buffer was previously demonstrated for some marine plants (Axelsson et al. 2000, Mercado et al. 2003).

CA activity

Biomass samples from laboratory cultures were incubated for 2 hours at 15º C and 200 µmol photons m^{-2} s^{-1} irradiance in Winkler flasks containing buffered ASW with 2mM total DIC at pH values of 6.5, 8, 8.5 and 9. These DIC concentrations and pH values roughly covers the conditions found in the cultivation tanks at the different water turnover rates. The CA activity of these samples was measured potentiometrically at 0-2º C, according to the method of Haglund et al. (1992a), using ca. 70 mg of biomass. Enzyme units were determined from the time taken for a linear drop of pH from 8.2 to 7, using the equation $((t_o/t_c)-1)/g$, where t_o and t_c are the times for pH changes of the noenzymatic and enzymatic reactions, respectively.

pH drift in closed vessel

The pH drift assays were performed inside a walk-in culture chamber providing constant temperature (15º C) and illumination (200 µmol photons m^{-1} s^{-1}) conditions. 1 g FW of *A. armata* from the laboratory cultures was inserted in a 110 mL Erlenmeyer containing natural seawater. The pH of the medium was previously adjusted to obtain an initial pH close to 6.5, by adding 0.1M HCl solution. The medium was continuously stirred with a magnetic bar. A plastic net placed between the bar and the sample avoided physical damage to the algae. A pH electrode (Crison GLP21, Alella, Spain) connected to a computer equipped with the Crison GLP21 software was inserted into the Erlenmeyer through a rubber stopper that prevented gas exchange with the air. The pH changes in the medium were recorded in the computer every 10 seconds. The experiment ended when the pH reached and maintained a stable value (±0.02) for at least one hour. This pH value represents the condition in which the DIC taken up by the algae equals the CO_2 released by respiration and/ or photorespiration into the medium. These experiments were also performed with the inhibitor of CA activity (AZ). Rates of pH change, expressed as ΔpH $(g\ FW)^{-1}$ min^{-1}, were calculated from the time-dependent pH variation and the biomass weight.

Results

Cultivation conditions

The concentration of dissolved inorganic carbon in seawater increased about 15% after passing through the fish tanks, with an associated decrease of the pH values from 8.17 ± 0.05

to 7.48 ± 0.03 (Fig. 1). After passing through the seaweed tanks this tendency was reversed both for DIC concentration and pH values. The decreases in DIC concentration and the increases of pH were more pronounced in seaweed tanks with lower water renewal rates and especially from noontime onwards as a result of a higher photosynthetic activity (Fig. 1a). During noon time (13:00) at the lowest water renewal rate tested (~0.1 vol h^{-1}), plants decreased the water DIC concentration from 2.81 ± 0.02 to 1.58 ± 013 mM, while at the highest (~4 vol h^{-1}), DIC concentration decreased to 2.46 ± 0.14 mM (Fig. 1a). Dissolved CO_2 was almost completely removed from the water by the plants in the lower water renewal tank and even in the highest water renewal tank, CO_2 concentration decreased 73% compared to the inflow values (from 66.6 ± 3.7 to 15.9 ± 7.4 μM). The DIC bulk use by the plants resulted in the increase of water pH after passing through the tanks, increasing from 7.48 ± 0.03 to 8.04 ± 0.19 in the highest water renewal tested and to 8.9 ± 0.16 in the lower one (Fig. 1b). Alkalinity values did not change after passing through the seaweed tanks (~2900 μmol L^{-1}).

Converting DIC concentrations to fluxes and plotting against the calculated DIC removal rates by the plants shows clearly that DIC removal increases with DIC availability (Fig. 2). A Michaelis-Menten function explains 91 % of the data variance. Its V_{max} is 0.12 mol DIC h^{-1} and the half saturation constant (K_s) is 0.31 mol DIC h^{-1}. The relationship between DIC availability and weekly growth is expressed by a similar function, with a V_{max} of 956 g DW m^{-2} wk^{-1} and a K_s of 0.19 mol DIC h^{-1} (Fig. 2).

Photosynthetic oxygen evolution experiments

The photosynthetic O_2 evolution of *A. armata* was insensitive to the addition of the buffers (data not shown). Figure 3 shows the measured rates of photosynthetic O_2 evolution

at different pH values. The highest values were measured at pH values ranging from 6.5 to 8, with no significant differences between them (~9 μmol O_2 gDW^{-1} h^{-1}). From pH 8 onwards, photosynthetic rates decreased sharply to minimum values lower than 1 μmol O_2 gDW^{-1} h^{-1}, at pH 9.5 and 10 (Fig. 3).

At natural seawater DIC (~2.2 mM) and pH (~8) conditions, O_2 evolution rates of *A. armata* were fully saturated (Fig. 4). At a pH 6.5, a DIC concentration of 0.4 mM was enough to meet the photosynthetic DIC demand, whereas at pH 8.5 the maximum photosynthetic rates were only achieved at a DIC concentration of 3 mM. At pH 9 the oxygen evolution rates were never saturated over the range of DIC concentrations used in this experiment (Fig.4). The addition of AZ in the reaction chamber equally depressed photosynthesis at all DIC concentrations for pH values of 8, 8.5 and 9, inhibiting photosynthesis by an overall mean of 29.73 ± 12.07 %, while at pH 6.5, AZ only inhibited photosynthesis (~30 %) at the lower DIC concentrations tested (0.2 and 0.4 mM). Figure 5 shows the inhibition percentage of O_2 evolution after adding AZ in all pH treatments incubated with 2 mM of DIC.

The estimated maximum photosynthetic rates (V_{max}) were not significantly different between the pH values tested, but the half saturation constant for total DIC ($K_{0.5}$) increased with pH (Table I). The addition of AZ in the reaction chamber decreased photosynthetic V_{max} and increased the half saturation constant values from pH 8 upwards.

CA activity measurements and pH drift

The external CA activity at pH 6.5 was significantly lower (2.34 ± 0.45 REA gFW^{-1}) than at pH values of 8, 8.5 and 9, showing a similar mean activity of 6.67 ± 2.25 REA gFW^{-1} (Fig. 5).

When cultured in a closed vessel, *A. armata* increased the pH of the medium up to 8.8 (Fig. 6). The pH compensation point decreased to 8.63 when AZ was added to the medium. The addition of the CA activity inhibitor also slowed down the rate of pH drift from around 40% at pH 8 to around 50% at higher pH values (Fig. 7). However, caution should be taken when interpreting these inhibition rates, as the AZ inhibitor influences the buffering capacity of seawater and thus the rates of the pH increase (Klenell et al. 2004).

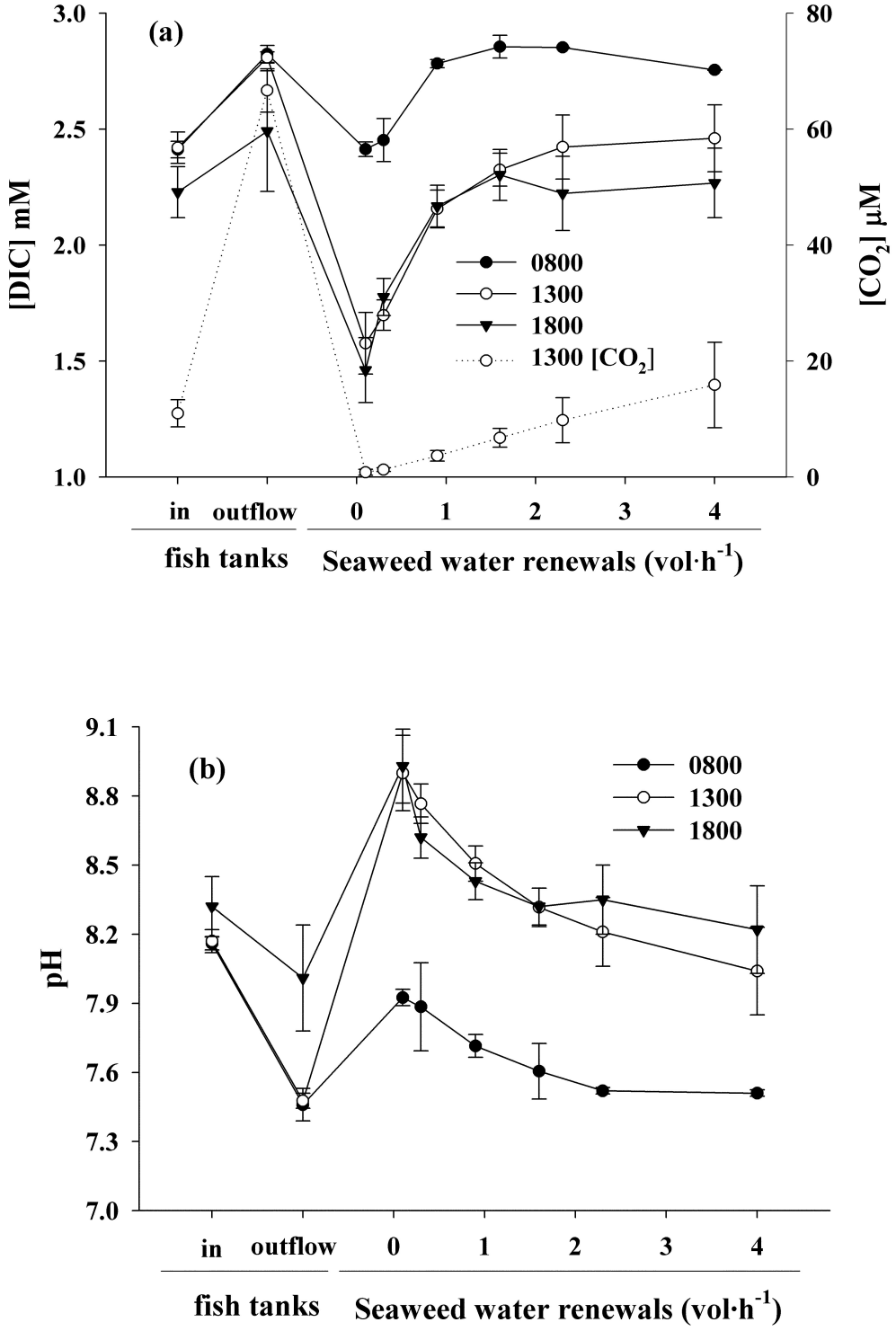

Fig.1 - DIC and CO_2 concentrations (a) and pH values (b) in the water before (fish tanks) and after passing through *Asparagopsis armata* tanks with different water renewal rates (vol. h^{-1}), at different times of the day (n=3).

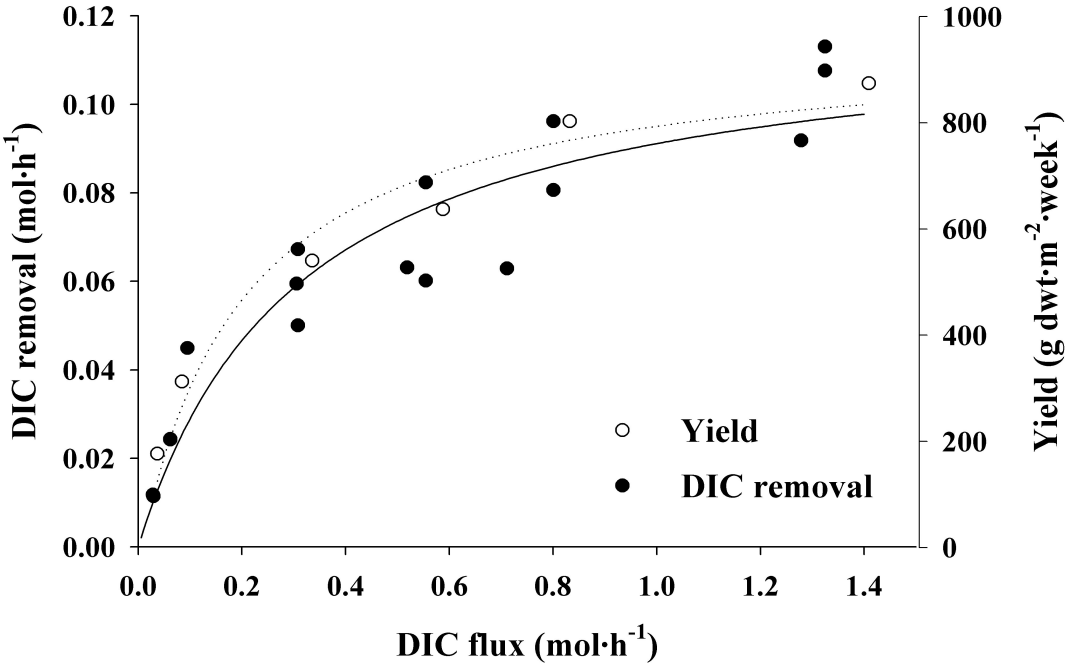

Fig. 2 – DIC removal at noon time (solid line) and weekly yield (dotted line) of *Asparagopsis armata* as a function of DIC fluxes.

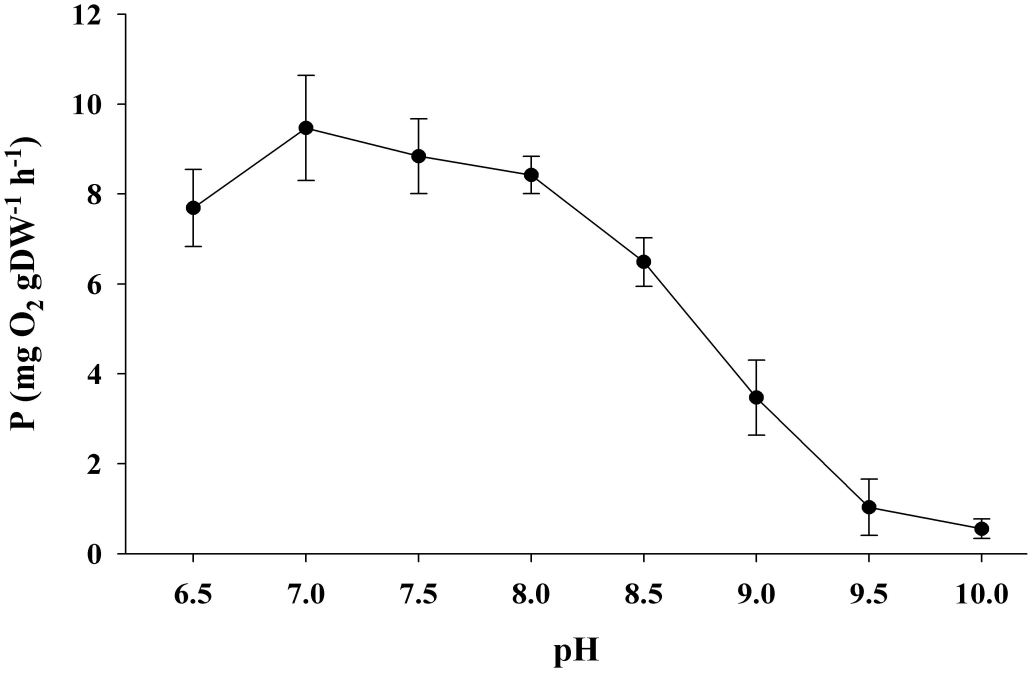

Fig. 3. Measured rates of photosynthetic O_2 evolution (P) as a function of pH for *Asparagopsis armata* in natural seawater (dissolved inorganic carbon [DIC] ~2.2 mM). Vertical error bars represent ± SD of the means (n = 4).

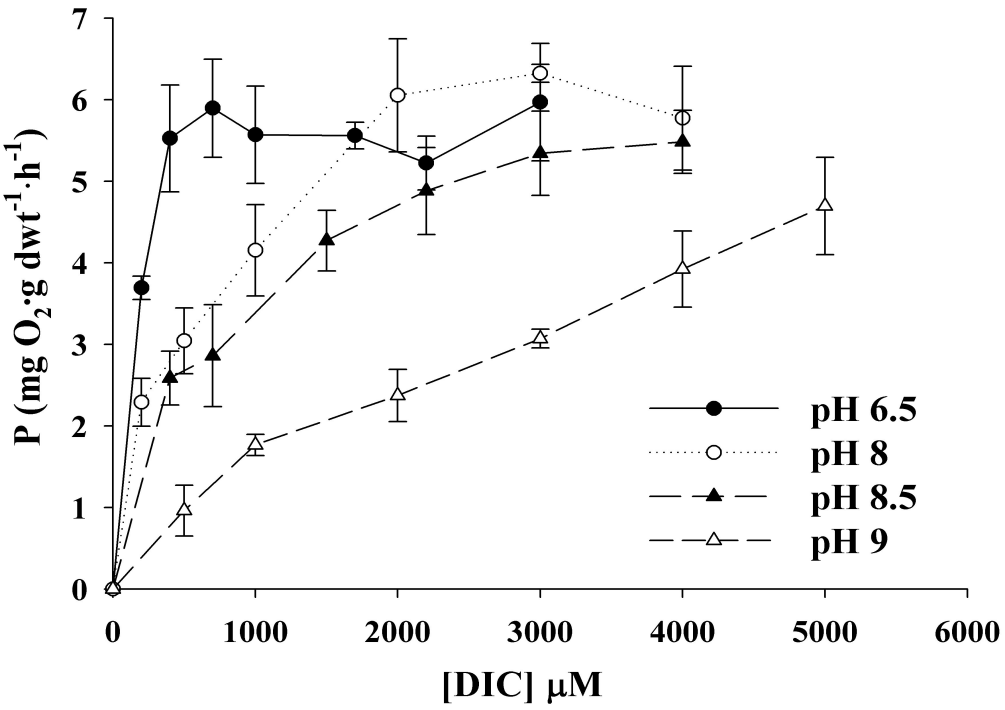

Fig. 4. Response of photosynthetic O_2 evolution rates of *Asparagopsis armata* to increasing total DIC concentration, at pH 6.5, 8, 8.5 and 9 (n=4).

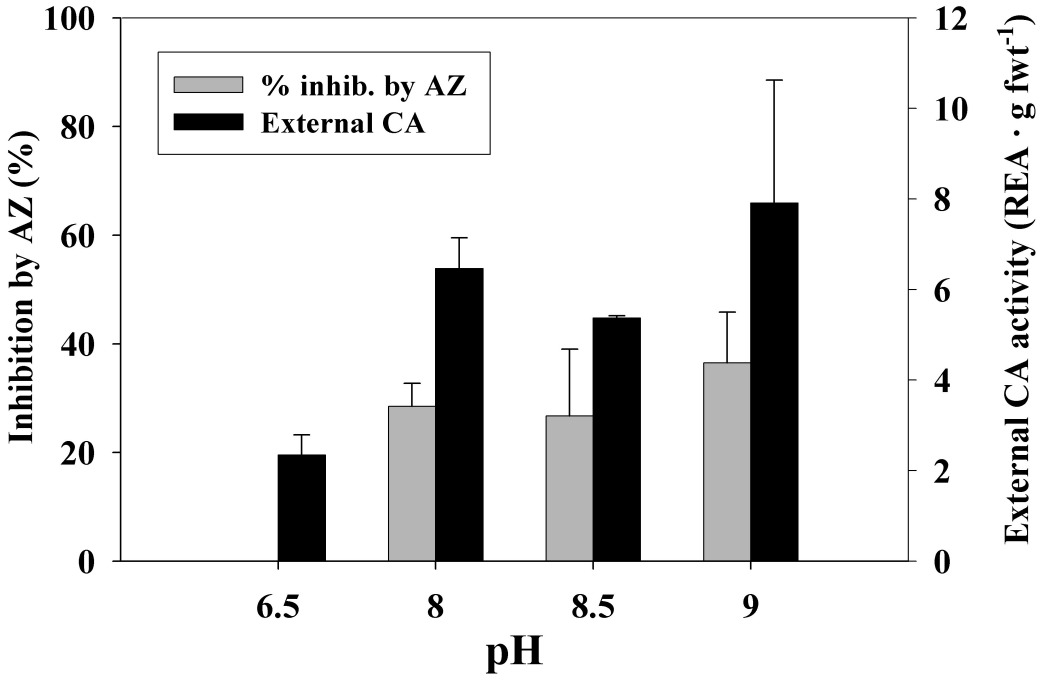

Fig. 5. Percentage of inhibition of photosynthetic O_2-evolution rates after the addition of acetazolamide (AZ) and external carbon anhydrase (CA) activity of *Asparagopsis armata* measured at pH of 6.5, 8, 8.5, and 9 at a dissolved inorganic carbon (DIC) concentration of 2 mM (n = 4). REA, relative enzyme activity.

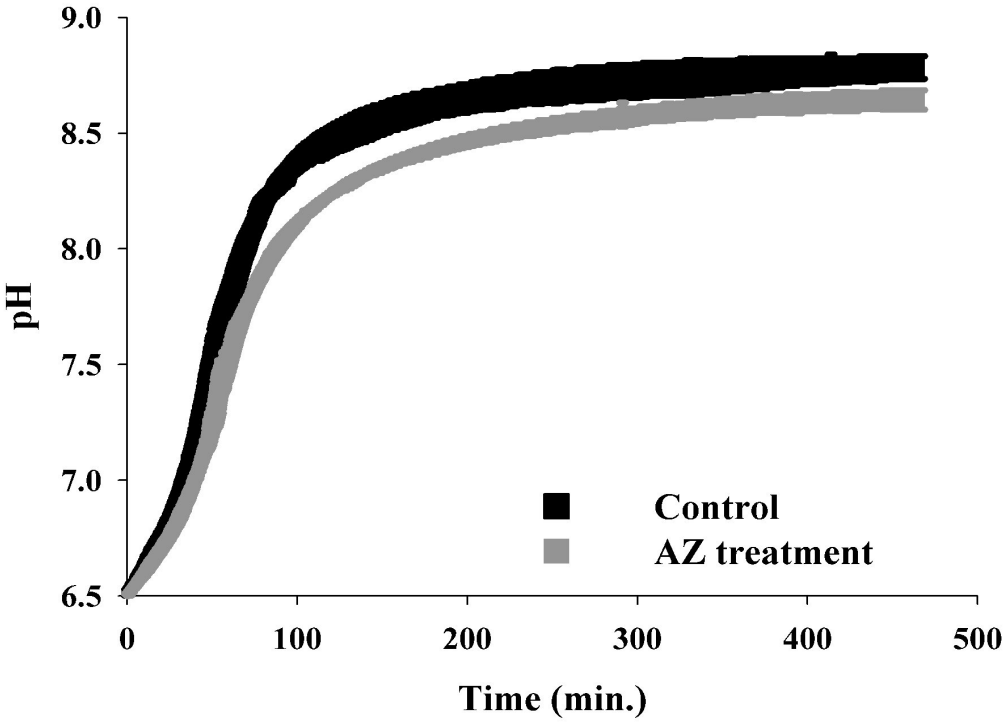

Fig. 6. pH drift experiments for *Asparagopsis armata* in natural seawater and in the presence of acetazolamide (AZ) for carbon anhydrase (CA) inhibition. The thickness of the curves represents the SD of three replicates.

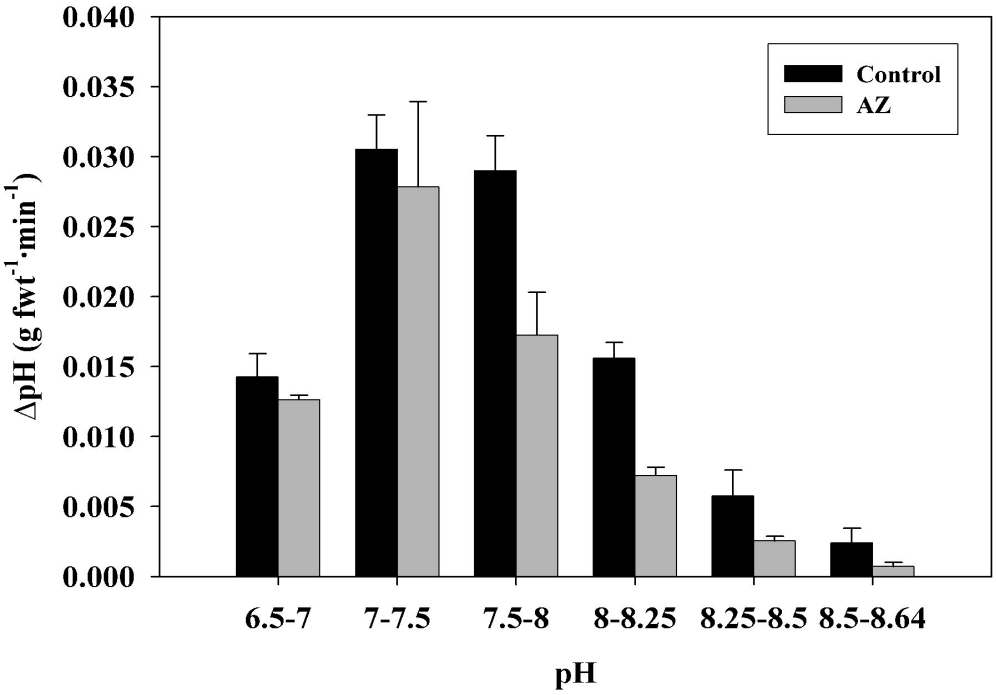

Fig. 7. Rates of pH drift, expressed as ΔpH (g FW)$^{-1}$ min^{-1}, at different external pH values for *Asparagopsis armata*. Data derived from pH drift experiments (n = 3).

Table 1. The dissolved inorganic carbon (DIC) maximum photosynthetic rate (V_{max}) and the concentration ($K_{0.5}$) supporting half of the V_{max} for *Asparagopsis armata* at four ambient pH values before and after adding the inhibitor AZ (n=4).

pH values	V_{max} (mg O_2 g DW^{-1} h^{-1})	$K_{0.5}$ Total DIC (mM)
6.5	5.83 ± 0.27	0.089 ± 0.03
6.5 +AZ	6.64 ± 0.43	0.294 ± 0.05
8	5.90 ± 0.37	0.336 ± 0.04
8 +AZ	4.50 ± 0.99	0.537 ± 0.20
8.5	6.07 ± 0.37	0.464 ± 0.11
8.5 +AZ	4.08 ± 0.41	0.611 ± 0.08
9	7.03 ± 2.31	3.384 ± 1.37
9 +AZ	5.67 ± 2.94	4.895 ± 2.99

Discussion

Fish-farm effluents may contribute the adequate amount of carbon for the photosynthesis requirements of high-density tank-cultivated *A. armata*. The contribution of CO_2 released from fish respiration shifted the equilibrium reactions of the different DIC forms in the water towards the right (see eq. 1), and consequently, the pH of the seawater available to the cultured plants decreased (from ~8.2 to ~7.45). In plants, photosynthetic uptake of CO_2 and/ or HCO_3^- results in a near stoichiometric production of hydroxyl ions, which increases pH (Cook et al. 1988, Prins and Elzenga 1989, Raven 1997). High water-renewal rates are thus necessary to maintain a low culture medium pH (i.e., as close as possible to that of the inflow water). Using the maximum water flow physically allowed by the tank design (~100 vol day^{-1}), resulted in a culture medium with DIC concentration above 2 mM and pH ~8 during the peak photosynthetic activity period. Under these cultivation conditions, *A. armata* was near its maximum rates of both carbon removal (V_{max}) and growth. Under similar laboratory conditions, *A. armata* attained its maximum photosynthetic O_2-production rates, meaning that this species has an identical dependence of photosynthesis and growth on the inorganic carbon supply. Reducing the water flow rates to the tanks until an almost stagnant water culture (0.1 vol h^{-1}), the pH increased to values near nine. A pH compensation point close to nine was also obtained for *A. armata* incubated in a closed vessel in the laboratory. This finding suggests that *A. armata* depends mainly on CO_2 for photosynthesis because at pH 9 the dissolved CO_2 is virtually absent and the uncatalyzed HCO_3^- dehydration rate is very slow to account for the photosynthetic requirements. At this point, species without a mechanism of HCO_3^- utilization reach their limit of DIC extraction capability (Cook et al. 1988, Maberly 1990, Johnston et al. 1992, Choo et al. 2002).

From the highest water renewal conditions to the lowest (from pH 8 to 9), the equilibrium concentration of CO_2 decreases ~90%, while HCO_3^- concentration decreases only about 30%. The steep decline of photosynthetic activity rates from pH 8 to 9 is also an indication that *A. armata* lacks an efficient mechanism for HCO_3^- utilization and relies mainly on free CO_2 to meet the photosynthetic needs (Prins and Elzenga 1989).

Despite all the indications of a low affinity for HCO_3^-, its use by *A. armata* was evidenced both from the potentiometric measurements and from the effects of AZ on photosynthesis and on the rates of pH drift. At pH values lower than 7.5, *A. armata* photosynthesis seems to rely solely on the CO_2 fraction of the DIC pool. At pH values higher than 7.5, the CO_2 supply to Rubisco seems to derive both from the diffuse entry of CO_2 and from the external dehydration of HCO_3^- mediated by CA. At pH 8, the contribution of this mechanism was essential for *A. armata* to attain maximum photosynthetic rates, compensating the lower availability of free CO_2. At higher pH values, the much lower availability of CO_2 was not compensated by an increase of the CA activity, and as a result, the CO_2 available to Rubisco was not enough to support high photosynthetic rates. This finding was also confirmed by the constant rates of inhibition by AZ on both photosynthesis and rates of pH drift at those pH values. The difference between the inhibition rates measured in the pH drift (between 40% and 50%) and in the photosynthetic rates (~ 30%) experiments, may be justified by the effect of AZ on the buffering capacity of seawater, which slows down the rate of pH increase (Klenell et al. 2004). On the other hand, the addition of a buffer in such low concentrations (200 μM), would have practically no inhibitory effect on photosynthesis due to its buffering capacity (Hellblom and Axelsson 2003). This means that the CA mechanism seems to be responsible for ~ 30 % of the total CO_2 fixed by the Rubisco in *A. armata*.

The simultaneous occurrence of CA with a low affinity for HCO_3^- use, has also been suggested for the gametophyte phase of *A. armata* (Mercado et al. 1998), even though the

mechanisms of DIC use are more habitat dependent than species dependent (Murru and Sandgren 2004). Low rates of CA activity in *A. armata* are consistent with the published values for subtidal red species and reinforce the described tendency for a reduced dependence on this mechanism in filamentous and deep-water species (Maberly 1990, Johnston et al. 1992, Larsson and Axelsson 1999).

We revealed that *A. armata* is a species with low HCO_3^- affinity. High water renewals within the cultivation tanks (~100 vol d^{-1}) is necessary to maintain the pH at low levels and thus supply enough carbon to maximize its growth. This need has a cost related to pumping. The reduction in the pumping costs in large-scale cultivation facilities can be attained but reduces growth due to increasing pH within the tanks. In the particular case of *A. armata*, it is possible, however, to optimize the pumping costs by reducing the water renewal rates from 4 to 2.5 vol h^{-1}, as it only decreases the maximum biomass production around by ~8%. This decrease does not have a significant effect in terms of ammonium biofiltration, if biofiltration is an objective of the integrated aquaculture (see Schuenhoff et al. 2006 - chapter 2).

The optimization of the pumping costs may be more cost effective in large scale cultivation of species with high affinity for HCO_3^-, as their biomass production is less sensitive to the medium pH and thus to the water renewal rates used. This was observed in *Ulva sp.*, a species with high affinity for HCO_3^- (Beer et al. 1990), when cultivated at the same conditions as *A. armata*. While *A. armata* biomass production was reduced in ~ 75% from the highest water renewal to the lowest, *U. rigida* growth was reduced only ~ 35% (Chapter 4).

This study revealed that seaweeds cultivated in fish farm effluents benefit not only from a rich source of ammonia but also from an important and free source of DIC for their photosynthesis. If supplied at high turnover rates fish farm effluents provide enough carbon

to maximize the photosynthesis of species with low affinity for HCO_3^-, preventing the artificial and costly supply of inorganic carbon to seaweed cultures.

CHAPTER 6

Tank cultivation of the seaweed species *Asparagopsis taxiformis* and *Bonnemaisonia hamifera* (Bonnemaisoniaceae) using fish pond effluents

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Abstract

The Bonnemaisoniaceae seaweed species are amongst those with the highest and broader spectrum of antimicrobial activity due to their high content and diversity of volatile halogenated compounds. These compounds can be extracted and explored to act as natural preservatives in cosmetics formulations. In this sense, the mass aquaculture of Bonnemaisoniaceae species should be considered as an opportunity. The aim of this work was to establish the cultivation in tanks of the tetrasporophyte phases of *Asparagopsis taxiformis* and *Bonnemaisonia hamifera* using the effluents of a fish farm. The seasonal variation of the species biomass production rates were compared with the previously investigated species, *A. armata*, which was cultivated at the same time under the same environmental conditions. The mechanisms of carbon use of both species were assessed as well to determine the cultivation conditions that maximize production.

The maximum yield values of *A. taxiformis* were recorded from April to July (~110 g DW m^{-2} d^{-1}), lower that yield values of *A. armata* (~127 g DW m^{-2} d^{-1}). Both *A. armata* and *A. taxiformis* did not survive the local summer temperatures values of 27 ºC and 29 ºC

respectively. The species *B. hamifera* was less productive. The highest biomass yield values for this species were registered in January (49.6 ± 0.96 g DW m^{-2} d^{-1}), lower than those measured for *A. taxiformis* (62.5 ± 1.92 g DW m^{-2} d^{-1}). Its culture was abandoned after five months because other species took over the cultures.

The biomass production of *A. taxiformis* became nutrient saturated when the cultures were supplied with 3 water exchanges per hour. Those conditions maintained the culture medium pH around 7.5 at the highest photosynthetic period of the day, essential to provide enough free CO_2 to meet the species photosynthetic needs. Both species showed to rely mainly on free CO_2 as dissolved inorganic carbon form for photosynthesis.

During the experimental period, from November to July, each square meter of the *A. taxiformis* integrated cultivation system produced about 24 kg DW. The total biofiltration service was 1.52 kg of N and 8.23 kg of C in the form of NH_4^+ and CO_2, respectively.

Introduction

Seaweed pecies belonging to the Bonnemaisoniaceae are rich sources of volatile halogenated compounds (VHCs) with remarkable antibacterial and antifungal activity (McConnell and Fenical 1977a, 1977b, 1980). Amongst several seaweed taxa screened, Bonnemaisoniaceae showed the strongest and broadest spectrum of antimicrobial activity (Pesando and Caram 1984, Ballesteros et al. 1992, Bansemir et al. 2006, Salvador et al. 2007). The VHCs of these algae are already explored by a company, which patented a special technique to extract the compounds to act as natural preservatives in cosmetics formulations, as anti–dandruff and scalp cleanser and as anti–acne treatment (Algues et Mer 2002). Besides VHCs, the species *Asparagopsis armata* produce sulphated galactans with promising therapeutic applications (Braun et al. 1983, Caporiccio et al. 1983) and new sources of anti-HIV compounds (Haslin et al. 2001). Considering all these chemical properties with potential market application, the mass aquaculture of Bonnemaisoniaceae species may be considered an economic opportunity.

We have previously established the tank cultivation of the tetrasporophyte phase of *A. armata* using the effluents of a fish farm in southern Portugal (Schuenhoff et al. 2006 – chapter 2). However, water temperatures above 24 ºC showed to be lethal for this species (Mata et al. 2006 – chapter 3) rendering such cultivation impossible during the summer in warm temperate regions. Here we assess the tank domestication of two other Bonnemaisoniaceae, *Bonnemaisonia hamifera*, distributed in Europe from Scandinavia in the north to the 25 ºC-summer isotherm in the south (van den Hoek 1982) and *Asparagopsis taxiformis*, a species distributed throughout the tropical and warm-temperate parts of the Atlantic and Indo-Pacific (Abbott and Williamson 1974, Price et al. 1986, Bonin and

Hawkes 1987). This species was recently detected in the Portuguese coast (Berecibar pers. comm.) and its provenience genetically confirmed to be from the Mediterranean lineage (Andreakis et al. 2007). Ní Chualáin et al. (2004) determined that the Mediterranean clade of *A. taxiformis* exhibited the wider range of survival and growth temperature limits of the genus, ranging from 9 to 31º C. To our best knowledge, the mass cultivation of *A. taxiformis* and *B. hamifera* was never tested before. The biomass production rates of both species are compared with the previously investigated species, *A. armata*, which was cultivated at the same time under the same environmental conditions.

The mechanisms of carbon use by both *A. taxiformis* and *B. hamifera* species were also studied to determine the TAN and DIC conditions that maximize *A. taxiformis* biomass production. By manipulating the effluent supply to the seaweed tanks both the total ammonia nitrogen (TAN) and the total dissolved inorganic carbon (DIC) levels, which highly influence the biomass production (see Schuenhoff et al. 2006 - chapter 2 and Mata et al. 2007 – chapter 5), may be adjusted. It is also critical to understand how selective the cultivated species are on the use of the different DIC forms (Mata et al. 2007). While some algae species are restricted to the passive diffusion of dissolved CO_2 for photosynthesis, others have developed mechanisms to use the most abundant DIC form in the medium (HCO_3^-) as an alternative source of carbon (Beer 1994, Larsson and Axelsson, 1999).

Materials and methods

Algae

Samples of the tetrasporophyte phase of *Asparagopsis taxiformis* from the Mediterranean were kindly provided from unialgal cultures at Stazione Zoologica, Naples, Italy (origin: Ischia, Naples, Italy). Samples of the *Bonnemaisonia hamifera* tetrasporophyte were obtained from the algal culture collection of the Martin Ryan Institute, National University of Ireland, Galway (*B. hamifera* culture no. 1027, origin: Ougella, Basque Country, Spain; leg. W.F. Farnham; voucher specimen: GALW015293). Samples of the tetrasporophyte phase of *A. armata* were collected in 2002 at Praia da Murração, Aljezur, Portugal and have been maintained in the laboratory at 15 ºC, 14:10 h L:D and 65 µmol photons m^{-2} s^{-1}. All three species were maintained and grown under these conditions, enriched with f/2 medium (Guillard and Ryther 1962), exchanged on a weekly basis.

Outdoor tank cultivation

Conditions and biomass up-scaling procedure

In October 2006, about 100 g FW of each of the three species was transferred from the laboratory stock culture to an integrated fish-seaweed cultivation unit at the Aquamarim aquaculture, southern Portugal. Seaweeds were inoculated in aerated cylindrical white (transparency ~70%) polyethylene tanks (Allibert Buckhorn, model C1100, Manterre - France; 110 L capacity, 0.23 m^2 surface area). The water for the seaweed cultures was pumped directly from one of the on growing fish tanks.

In an initial phase, the tanks were shaded with a net to reduce the irradiance levels, as photoinhibition of *A. armata* occurs at a biomass density lower than 5 g FW L^{-1} (Mata et al. 2006 – chapter 3). We assumed 5 g FW L^{-1} as the ideal stocking density for *A. taxiformis* and *Bonnemaisonia hamifera* species, based on the results previously obtained for *A. armata* (Schuenhoff et al. 2006 – chapter 2, Mata et al. 2006 – chapter 3). When the biomass density of 5 g FW L^{-1} was exceeded inside the tanks, the excess biomass and the shade net were transferred to another tank. This up-scaling process was continued until all the experimental units were stocked.

Experimental design

Two tanks per species, supplied with water flow rates above 4 tank turnovers per hour (vol h^{-1}), were used to monitor the seasonal variation of the biomass yield of the three species. Previous results on *A. armata* showed that at these high flow rates growth is not limited by carbon and/or nitrogen (Schuenhoff et al. 2006 – chapter 2, Mata et al. 2007 – chapter 5). Four more tanks were used to assess the effects of a wide range of water flow rates (between 0.2 and 6 vol h^{-1}), and thus of associated TAN and DIC fluxes (water renewal rates x TAN and DIC concentrations), on the biomass yield of *A. taxiformis*. The water renewal rate threshold of non-limiting nutrient conditions was determined as the level at which the increment in the biomass yield with water flow decreased to less than 15%. Both experiments were monitored on a monthly basis, from November 2006 onwards. Every month, the water flow rates to the tanks were carefully monitored at midday, during one week. The pH, temperature and salinity of the fish effluents were measured and two water samples were collected and taken to the laboratory to determine alkalinity and TAN concentration. The water pH within each tank was monitored as well. The DIC concentration

was calculated from the pH, alkalinity, salinity and temperature values using the software developed by Lewis and Wallace (1998).

The biomass yield, which represents the rate of biomass accumulation during the experimental week, was calculated using the equation:

$$Y\ (g\ DW\ m^{-2}\ d^{-1}) = [(N_t - N_0)/t/A] \cdot (DW/FW),$$

where, N_t is the final fresh weight, N_0 the initial fresh weight, t the number of days, A the area covered by the tank and DW/FW the dry weight/fresh weight ratio. The seaweed fresh weight was determined by centrifuging the biomass in nylon bags (0.1 mm mesh) at 2800 rpm to constant weight, using a domestic spin dryer. The dry weight was obtained by oven-drying 10 centrifuged samples during 48 h at 60 °C. The DW/FW relationship was about 0.25 for all species.

At the end of each week, two seaweed samples were taken from each of the two tanks supplied with water renewal rates of 4 vol h^{-1}, for tissue C and N analysis. The amount of nitrogen and carbon incorporated into the new biomass, and thus removed from the fish effluents, was estimated multiplying the elemental tissue content by the biomass yield.

The open-air photon flux density (PFD) was monitored using a Li-190SA Quantum Sensor connected to a Li-250A Data Logger (both LI-COR Inc., Lincoln, Nebraska, USA). The pH, temperature and salinity were monitored using a pH probe (YSI, model 63, Yellowsprings, Oiho, USA). A whole week time series of temperature was obtained placing a temperature logger (MAXIM, iButton model DS1921G, Sunnyvale, CA, USA) inside one of the tanks. Water samples for TAN and alkalinity analysis were filtered with 0.25 µm CF Whatman acetate filters and immediately taken to the laboratory at 4º C. TAN concentration was measured on a loop-flow analyser (uMAC-1000 multiparametric, Systea, Anagni, Italy) using the standard indophenol blue procedure described in Grasshoff (1983). Alkalinity was calculated from linear Gran-plots (Gran 1952) after potentiometric titration of 137 mL

sample with 0.05 N HCl (Bradshaw et al. 1981). Biomass samples for tissue C and N determination were oven-dried (48 h; 60 ºC) and reduced to a fine powder prior to be analysed in an elemental analyser (Flash EA 1112, ThermoFinnigan).

A multiple linear regression model of *A. taxiformis* production was developed to assess the effects of temperature, irradiance and TAN and DIC supply (independent variables) on biomass yield (dependent variable), using all data collected along the production experiments.

Carbon use

Photosynthetic oxygen evolution experiments

To evaluate the mechanisms of carbon use by the species, we first tested the effects of pH on the photosynthetic O_2 evolution. Samples of 10 to 20 mg FW of *A. taxiformis* and *B. hamifera* were incubated in a Clark type oxygen electrode (DW3 measuring chamber, Hansatech Instruments, Norfolk, UK). A slide projector provided an irradiance of 160 µmol photons m^{-2} s^{-1} to the chamber (the irradiance for maximum photosynthesis determined for *A. armata*; Mata et al. 2006 - chapter 3), which contained 15 mL of natural seawater maintained at 15º C and buffered at the selected pH values. A known amount of biological solid buffer (Sigma) with a pK_a appropriate to the experimental pH was added to provide a final concentration of 25 mM. The pH was then adjusted as desired with freshly prepared 1M NaOH and HCl solutions. The buffers used were Mes for pH 6.5, Hepes for pH 7 and 7.5, Tris for pH 8, 8.5 and 9 and Caps for pH 9.5 and 10. No inhibitory effects of the buffers on photosynthesis were observed.

Carbonic anhydrase activity experiments

The activity of the periplasmic carbonic anhydrase (CA), which mediates the dehydration of HCO_3^- into CO_2 that can then be transported into the cell (Haglund et al. 1992a, Mercado et al. 1998, Larsson and Axelsson 1999), was tested in both *A. taxiformis* and *B. hamifera* to infer if the species can use bicarbonate (HCO_3^-) as an alternative source of carbon for photosynthesis. The CA activity was measured potentiometrically at 0-2º C, according to the method of Haglund et al. (1992a), using ca. 70 mg of biomass from the laboratory cultures. Units of relative enzyme activity (REA) were determined from the time taken for a linear drop of pH from 8.2 to 7, using the equation $((t_o/t_c)-1)/g$, where t_o and t_c are the times for pH changes of the noenzymatic and enzymatic reactions, respectively.

pH drift in closed vessel

A portion of 1 g FW of each *A. taxiformis* and *B. hamifera* were incubated in sealed Erlenmeyers containing 110mL of natural seawater at 15º C and irradiated with 200 μmol photons m^{-2} s^{-1}. The medium was continuously stirred with a magnetic bar. A plastic net placed between the bar and the sample avoided physical damage to the algae. A pH electrode (Crison GLP21, Alella, Spain) connected to a computer equipped with the Crison GLP21 software was inserted into the Erlenmeyer through a rubber stopper that prevented gas exchange with the air. The pH changes in the medium were recorded in the computer every 10 seconds. The experiment ended when the pH reached and maintained a stable value (±0.02) for at least one hour (pH compensation point). This pH value represents the condition in which the DIC taken up by the algae equals the CO_2 released by respiration and/ or photorespiration into the medium.

Results

Outdoor tank cultivation

All three species adapted successfully to the outdoor tank cultivation conditions. It took six weeks to upscale the initial stock of 100 g FW of each species to two tanks with the desired stocking density (5 gFW L^{-1}). The experiments started in November and ran until July.

Abiotic conditions

The water temperature within the tanks varied from a minimum of 9 ºC in December to a maximum of 29 ºC in July (Fig. 1). Coinciding with the minimum December temperature, the fish effluent showed the lowest TAN concentration (15.5 ± 3.8 µM) and the highest pH value (7.37 ± 0.24) (Fig. 2). These conditions changed progressively towards spring, i.e. TAN concentration increased while pH decreased. The lowest pH values were recorded in March (6.63 ± 0.08) whereas the TAN concentration of the fishpond effluent peaked in April (71.4 ± 6.2 µM). In May, an electricity failure occurred resulting in fish mortality and from that point onwards the TAN concentration of the fish effluents decreased to initial spring values of 30 to 40 µM (Fig. 2).

Seasonal variation of the maximum biomass production

Asparagopsis armata was the most productive species during the whole experimental period. Its biomass yield increased from November (58.2 ± 1.3 g DW m^{-2} d^{-1}) to April (127

± 4.8 g DW m^{-2} d^{-1}), stabilizing until the end of June, when maximum water temperatures of 27.5 ºC (daily mean of 24 ºC) were recorded in the tanks and some individuals started to bleach. In July the cultivation ceased. *A. taxiformis* was the species that kept in culture for a longer period, but it did not survive maximum water temperature values of 29 ºC (daily mean of 25.5 ºC) recorded in the end of July. Maximum biomass yield values of ~110 g DW m^{-2} d^{-1} were maintained from April to July. The species *Bonnemaisonia hamifera* was the least productive of the three species. Its highest yield values registered in January (49.6 ± 0.96 g DW m^{-2} d^{-1}) were significantly lower than those measured for *A. taxiformis* (62.5 ± 1.92 g DW m^{-2} d^{-1}) in the same period of time. Its cultivation was abandoned in March due to a serious infestation of *Ulva* spp. In contrast, the cultures of *Asparagopsis* species developed almost free of other seaweed species until May. At this time, some contamination of *Ulva* spp occured but it stayed at low levels. Only during the decay period of target species the *Ulva* spp. developed at significant levels.

The seasonal variation of the biomass production of *A. taxiformis* species may be predicted ($P<0.001$ and $R=0.89$) from a linear combination of the independent variables through the equation: Biomass Yield = -33.41 + (4.35 * DIC flux) + (0.048 * TAN flux) + 0.99 * Temp) + (0.061 * Irrad). DIC fluxes and irradiance levels are the variables that appear to account mostly for the ability to predict yield (Table 1).

Nitrogen and carbon removal rates

The tissue N and C content was similar among the three species, with overall values ranging from 5.9 % to 6.9 % and from 33% to 37% respectively. The species *A. armata* was in general the most efficient biofilter, removing a minimum of 3.7 g of N m^{-2} d^{-1} and 19 g of C m^{-2} d^{-1} in November and a maximum of 7.9 g of N m^{-2} d^{-1} and 43.1 g of C m^{-2} d^{-1} in April

(Table 2). The highest removal rates were registered in July, when *A. taxiformis* removed 8.1 g of N m^{-2} d^{-1} and 44 g of C m^{-2} d^{-1}. *Bonnemaisonia hamifera* showed the lowest removal rates, ranging from 1.8 g of N m^{-2} d^{-1} and 9.3 g of C m^{-2} d^{-1} in December to 3.2 g of N m^{-2} d^{-1} and 17.6 g of C m^{-2} d^{-1} in January (Table 2).

Effects of water renewal rates on *Asparagopsis taxiformis* yield

The biomass yield of *A. taxiformis* increased asymptotically with the amount of water supplied to the tanks (Fig. 3). The rate of biomass yield increase with water renewal was generally lower in winter, increased to the spring and was highest in July.
The water renewal threshold for biomass yield saturation was about 3 vol h^{-1} in every month of the experiment (Table 3). The saturation levels for the associated fluxes of TAN and DIC are also presented in Table 3. A wider range of TAN saturation values was observed. In December, the *A. taxiformis* biomass production tended to saturation at a lowest of 46.4 μM TAN h^{-1}, whereas the highest TAN saturation level was observed in April (219 μM TAN h^{-1}, Table 2). The relationship of biomass yield with both DIC fluxes and pH revealed a narrower limitation / saturation window for *A. taxiformis* growth. Saturation occurred between 8.6 and 10 mM DIC $h^{-1}$and at pH levels between 7.42 in June and 7.81 in December (Table 3).

Carbon use

The response pattern of the photosynthetic O_2 evolution rates to different pH values was identical in all three species (Fig. 4). Highest production rates were registered at pH values ranging between 6.5 and 8, beyond which they decreased sharply. The absolute

photosynthetic rates of both *Asparagopsis* species were similar and significantly higher than those of *B. hamifera*.

The detected CA activity in *A. taxiformis* (6.48 ± 3.8 REA g FW^{-1}) was very similar to the activity previously measured in *A. armata* (6.67 ± 2.3 REA g FW^{-1}), while in *B. hamifera* the enzyme activity was negligible (Table 4). The pH-drift experiment revealed similar pH compensation points for all three species (around 8.8).

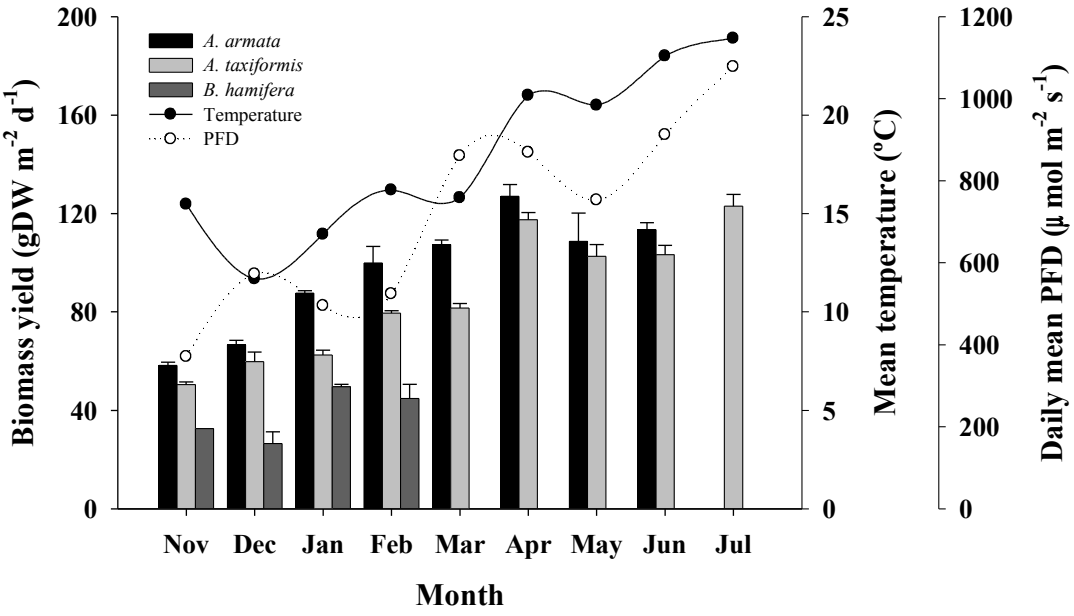

Fig. 1 – Seasonal variation of the biomass yields of *Asparagopsis armata*, *A. taxiformis* and *Bonnemaisonia hamifera*. The lines represent averages of water temperature (continuous line) and daily photon flux density (PFD; dotted line).

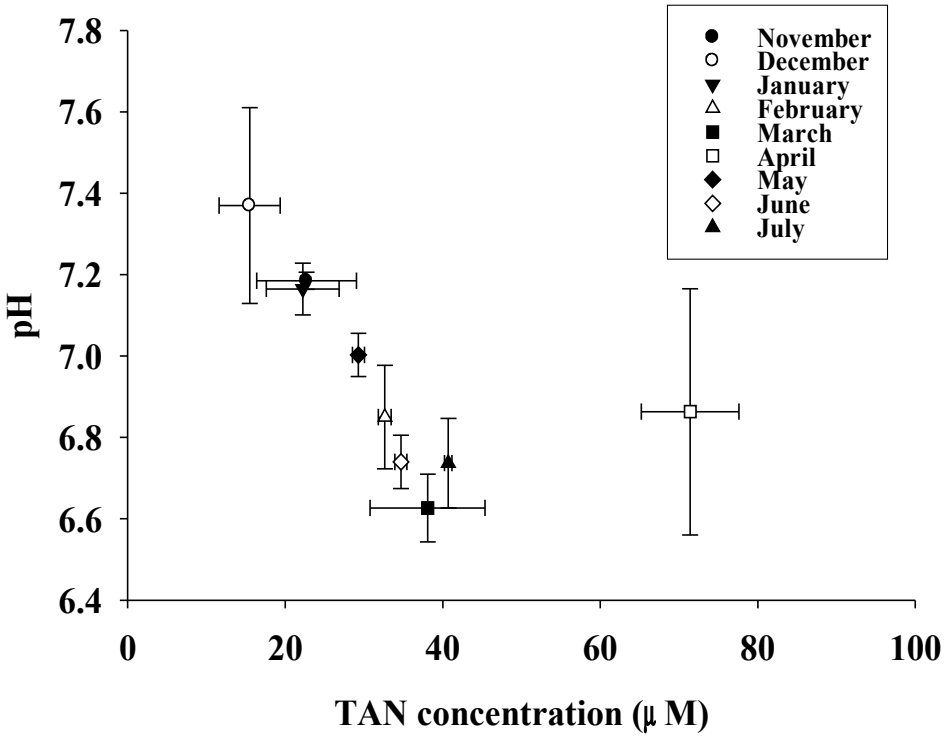

Fig. 2 –Mean TAN concentration and pH of the fishpond effluent available to the seaweed cultivation system.

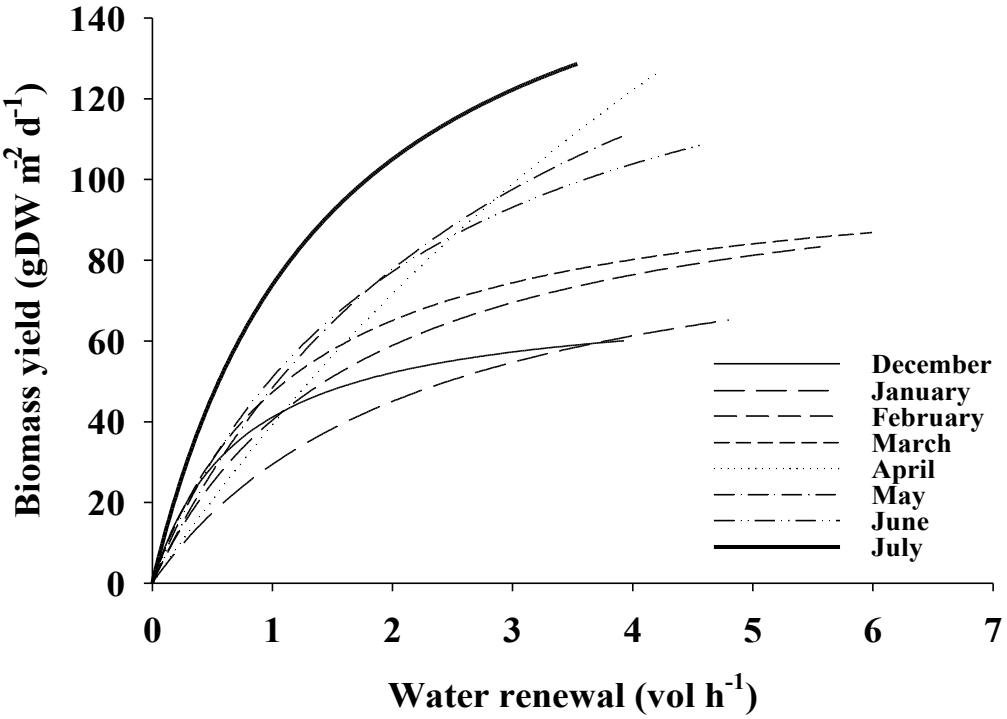

Fig. 3 – Monthly variation of the effects of water renewal on the biomass yield of *Asparagopsis taxiformis*.

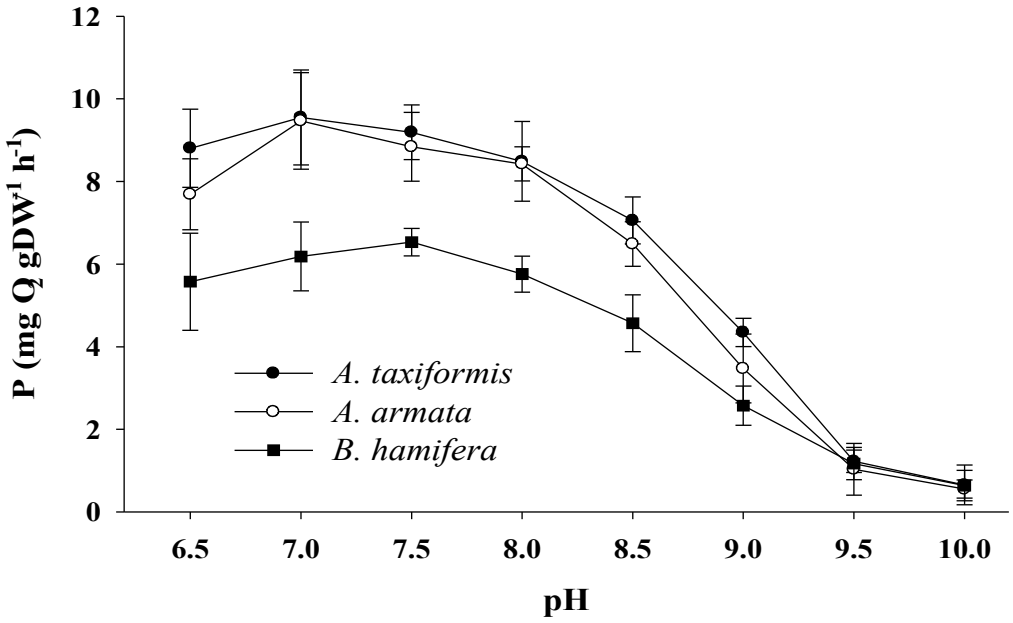

Fig. 4. Rates of photosynthetic O_2 evolution (P) as a function of pH for *Bonnemaisonia hamifera*, *Asparagopsis taxiformis* and *A. armata* (this species' data from Mata et al. 2007 - chapter 5) in natural seawater (DIC ~2.2mM). Vertical error bars represent the standard deviation of means

Table 1 – Results of the multiple linear regression applied to the *A. taxiformis* yield data (dependent variable) with temperature, irradiance and TAN and DIC supply as independent variables.

	Coefficient	Std. Error	t	P
Constant	-33.413	10.61	-3.147	**0.003**
DIC flux	4.35	0.837	5.196	**<0.001**
TAN flux	0.048	0.063	0.759	0.452
Temperature	0.986	1.052	0.937	0.354
Irradiance	0.061	0.021	2.871	**0.006**

Table 2 – Monthly removal rates of N and C from the fish effluent by the species *Asparagopsis taxiformis Bonnemaisonia hamifera* and *A. armata*.

Species	*A. taxiformis*		*B. hamifera*		*A. armata*	
Month	N removal ($g\ m^{-2}\ d^{-1}$)	C removal ($g\ m^{-2}\ d^{-1}$)	N removal ($g\ m^{-2}\ d^{-1}$)	C removal ($g\ m^{-2}\ d^{-1}$)	N removal ($g\ m^{-2}\ d^{-1}$)	C removal ($g\ m^{-2}\ d^{-1}$)
November	3.27 ± 0.06	17.8 ± 0.1	2.18 ± 0.01	11.31 ± 0.4	3.7 ± 0.03	19.03 ± 0.3
December	3.74 ± 0.23	19.88 ± 1.1	1.76 ± 0.34	9.34 ± 1.9	4.29 ± 0.09	23.29 ± 0.4
January	3.79 ± 0.57	20.84 ± 3.2	3.22 ± 0.13	17.61 ± 0.7	5.57 ± 0.01	30.82 ± 0.1
February	5.43 ± 0.03	29.04 ± 0.4	3.08 ± 0.37	16.64 ± 2.3	6.49 ± 0.16	35.32 ± 1.3
March	5.19 ± 0.09	28.69 ± 0.4			6.93 ± 0.03	37.74 ± 0.1
April	7.15 ± 0.45	39.82 ± 1.3			7.93 ± 0.13	43.09 ± 0.4
May	5.52 ± 1.01	29.7 ± 5.7			6.53 ± 0.28	35.7 ± 2.4
June	5.56 ± 0.05	31.06 ± 0.1			7.04 ± 0.31	37.45 ± 1.8
July	8.07 ± 0.51	43.96 ± 1.4				

Table 3 – Monthly saturation thresholds of *Asparagopsis taxiformis* biomass yield.

	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July
Water renew. ($vol\ h^{-1}$)	2.9	2.9	2.8	2.9	3.1	3.0	3.0	2.8
TAN flux ($\mu M\ h^{-1}$)	46.4	67.1	92.4	112.2	219.0	89.9	88.7	87.5
DIC flux ($mM\ h^{-1}$)	8.6	8.7	8.8	9.3	10.0	9.8	9.6	9.0
pH	7.81	7.76	7.49	7.46	7.52	7.40	7.42	7.52

Table 4 - External CA activities and pH compensation points of *Asparagopsis taxiformis, Bonnemaisonia hamifera* and *A. armata* (data of *A. armata* from Mata et al, 2007 - chapter 5).

CA activity (REA g^{-1} FW)			pH compensation point		
A. taxiformis	*B. hamifera*	*A. armata*	*A. taxiformis*	*B. hamifera*	*A. armata*
6.48 ± 3.8	1.09 ± 0.9	6.67 ± 2.3	8.79 ± 0.03	8.77 ± 0.06	8.8 ± 0.08

Discussion

The two novel candidates for mass seaweed aquaculture, *Asparagopsis taxiformis* and *Bonnemaisonia hamifera* adapted well to the tank cultivation conditions. While *Bonnemaisonia hamifera* was much less productive than *A. armata*, *A. taxiformis* production was just lower. Similarly to *A. armata*, this species presented biomass yield values higher than *Ulva rigida* cultivated in this system (chapter 4).

The cultures of both *Asparagopsis* species remained free of nuisance species during almost all the experimental period. A sudden development of ulvoid thalli observed in May was most probably related with an electricity failure that resulted in the lack of aeration within the tanks during a whole night and morning of the following day. We showed elsewhere that such events result in the degradation of *A. armata* physiological conditions (Figueroa et al. 2006) that eventually lead to the culture crash. The individuals may recover some days after reestablishing the normal cultivation conditions (Figueroa et al. 2006), but in the meantime opportunistic green species may develop. The *B. hamifera* cultures were early contaminated with other seaweed species and after 5 months they were severely contaminated. The control of nuisance species is a common problem in red seaweed aquaculture (Bidwell et al. 1985, Friedlander 1992, Demetropoulos and Langdon 2004). It is usually performed by managing the biomass density and thus the light within the tanks (see Bidwell et al. 1985). The biomass density used in our experiments was selected based on previous results for *A. armata* (Schuenhoff et al. 2006 – chapter 2). Higher stocking biomass densities probably have to be used to cultivate *B. hamifera* free of nuisance species.

The photosynthetic rates of the species confirmed *B. hamifera* as the least productive of all the three Bonnemaisoniaceae, but their photosynthetic responses to different pH values presented a similar pattern, with a steep decline of the photosynthetic rates from pH 8 and 9.

This is an indication that the three species rely mainly on free CO_2 to meet their photosynthetic needs. From pH 8 to 9 the equilibrium concentration of CO_2 decreases ~90%, while the HCO_3^- concentration decreases only about 30% (Prins and Elzenga 1989). The carbon use mechanisms of *A. taxiformis* and *B. hamifera* are similar to *A. armata*, previously described in Mata et al (2007 - chapter 5). The pH compensation points below 9 further confirmed the apparent low affinity for HCO_3^- uptake by the species. At pH 9 the dissolved CO_2 is virtually absent and the uncatalyzed HCO_3^- dehydration rate is very slow to account for the photosynthetic requirements, so species without a mechanism of HCO_3^- utilization reach their limit of DIC extraction capability (Cook et al. 1988, Maberly 1990, Johnston et al. 1992, Choo et al. 2002). Furthermore, the measured activity of the enzyme responsible to mediate the dehydration of HCO_3^- into CO_2 (CA enzyme) was relatively low in *A. taxiformis* and almost absent in *B. hamifera*.

The production of species that depend almost exclusively on dissolved CO_2 for photosynthesis may be more susceptible to C than to N availability (see Rivers and Peckol, 1995). Evidence of the importance of DIC flux on the *A. taxiformis* production is revealed by the biomass yield production model. The biomass yield variations were more predicted by the DIC fluxes than by any other abiotic parameter used in the model (Table 1). Another indication is revealed by the relationships between biomass yield and the different water parameters (Table 3). The threshold DIC fluxes and pH values were more consistent throughout the experimental months than the TAN fluxes threshold values. As concluded for *A. armata* (Mata et al. 2007 - Chapter 5), the cultivation of these species is dependent on a proper supply of CO_2, probably their main limiting factor for biomass production. In this integrated cultivation system, optimum conditions were attained when fish farm effluents were supplied to the seaweed tanks at a minimum of 3 vol h^{-1}, maintaining the pH of the cultures at around 7.5 (at midday). This is in well accordance with the laboratory

photosynthetic experiments, which showed that only mediums with pH values around or below 7.5 had enough CO_2 to saturate *A. taxiformis* photosynthesis.

Both *Asparagopsis* species presented similar seasonal patterns of biomass yield variation. The seasonal fluctuations of *A. taxiformis* yield in the tanks supplied with enough TAN and CO_2 were more related to irradiance than to temperature. However, the local summer temperature caused the culture crash. As initially predicted, *A. taxiformis* persisted longer through the summer than *A. armata*. A temperature of 31 ºC was previously described as the lethal limit for this species clade (Ní Chualáin et al. 2004). In this integrated fish/seaweed cultivation system it is not possible to maintain a continuously year round production of these species, but the data gathered can be used to predict the year-round productivity in a similar cultivation system located elsewhere, where the maximum water temperature in the seaweed tanks does not surpass 29 ºC. In such a system, each square meter of the *Asparagopsis taxiformis* integrated cultivation system would produce every year about 37 kg DW (148 kg FW) of biomass, removing from the fish farm effluent about 1.83 kg of N and 9.23 kg of C in the form of NH_4^+ and CO_2, respectively. Should future legislation pressure the aquaculture industry to internalize environmental costs for their waste discharge, N and C biofiltration may turn from just a by-product of seaweed production into an economic benefit to the producer.

CHAPTER 7

Effects of carbon, nitrogen and hydrogen peroxide availability on major volatile halogenated compound content in the red alga *Asparagopsis taxiformis* (Bonnemaisoniaceae)

Abstract

Volatile halogenated compounds (VHC), while rare in terrestrial plants are common in marine algae. *Asparagopsis* species contain a high variety and quantity of these secondary metabolites, which are already extracted and used in cosmetics formulations. Considering the economic potential of the *Asparagopsis* biomass, we have established a tank-cultivation system for their mass cultivation in a fish farm of southern Portugal. After establishing the optimal cultivation conditions to maximize the biomass production it is important to determine which conditions maximize the VHC content in the produced biomass. So far there is little information on the production mechanisms of the vast number of VHC and how environmental factors affect the VHC content in algae. With this study we aim to assess if and how it is possible to increase the internal levels of the major VHC (bromoform and dibromoacetic acid) of *Asparagopsis taxiformis* in culture and how it affects the biomass growth. The effects of hydrogen peroxide (H_2O_2), of TAN and of CO_2 on the halogenated compound concentration were tested in the laboratory. Furthermore, the effects of TAN and

CO_2 on the *A. taxiformis* halogenated metabolites content were investigated in the *Asparagopsis* spp.-integrated aquaculture system.

The addition on the culture medium of H_2O_2, a substrate involved in the bromoform production, resulted in a decrease of both bromoform and DBA in *A. taxiformis* tissue. We speculate about the enzyme (haloperoxidase) prosthetic group to explain this tendency. Whereas the availability of CO_2 resulted in higher bromoform content of *A. taxiformis* tissue, the availability of TAN had the opposite effect. In integrated aquaculture, the supply of higher water renewals provides higher TAN and CO_2 to the seaweeds. Results showed that the bromoform content in *A. taxiformis* is more influenced by the CO_2 availability than by the TAN availability. At CO_2 limiting conditions for the species photosynthesis both growth and production of carbon based secondary compounds were carbon limited. Therefore in integrated fish/seaweed aquaculture, the nutrient conditions that maximize the biomass yield increase as well the bromoform content in the produced biomass.

Introduction

The species of the red algal family Bonnemaisoniaceae produce a wide variety of halogenated metabolites (Fenical, 1975; McConnell and Fenical, 1977a; 1977b; 1980, Rose et al. 1977). In the genus *Asparagopsis* alone, over 100 volatile halogenated compounds (VHC) are found, including haloforms, haloacids and haloketones (McConnell & Fenical 1977a, Woolard et al. 1979), which are concentrated in specialised structures known as gland cells (Wolk 1968, Fenical 1975, Marshall 2003, Paul et al. 2006a, b). The high diversity and quantity of secondary compounds confer to the *Asparagopsis* species extracts a remarkable antifouling activity. In comparison with other seaweed taxa they usually show the strongest and broadest spectrum of antimicrobial activity (Hornsey and Hide 1974, Pesando and Caram 1984, Reichelt and Borowitzka 1984, Ballesteros et al. 1992, Bansemir et al. 2006, Salvador et al. 2007). So far, the production mechanisms of the vast number of VHC produced by algae are still elusive, but the formation of the major compounds such as bromoform appear to be related with oxidative stress involving the hydrogen peroxide (H_2O_2) and the haloperoxidase enzyme (Wever et al. 1991, Collén et al. 1994, Sundström et al. 1996, Pedersén et al. 1996, Manley & Barbero 2001). The enzyme catalyzes the oxidation of halides ions (X^-: iodide, bromide and chloride) by H_2O_2, resulting in the halogenation of certain organic substrates (Butler and Walker 1993):

$$X^- + H_2O_2 + R\text{-}H + H^+ \rightarrow R\text{-}X + 2\ H_2O$$

According to Reichardt et al. (1991), the concentrations of the precursor molecules are the most important determinant of the rate of secondary metabolite production. The addition of enzyme substrates to the medium increased the haloperoxidase activity and the VHC production (Wever et al. 1991, Collén et al. 1994,

Sundström et al. 1996, Pedersén et al. 1996, Manley & Barbero 2001, Ohsawa et al. 2001). Most of the studies concluded that the supply of H_2O_2 was the most important rate-determining step for bromoform formation (e.g. Ohsawa et al. 2001).

Considering the economic potential of the *Asparagopsis* biomass, we have established a tank-cultivation system for their mass cultivation in a fish farm of southern Portugal (Schuenhoff et al. 2006 – chapter 2, chapter 5). In previous chapters, the nutrient (nitrogen and carbon) conditions that optimise both the biomass production and the nutrient biofiltration were determined, but these might not be the same conditions that optimize the concentrations of secondary metabolites. The availability of carbon and nitrogen in the environment is expected to control the concentration of secondary metabolites in plant tissues, according to the carbon-nutrient balance (CNB) hypothesis (Bryant et al. 1983). However, this and most of the plant defence model theories assume reduced growth at conditions that increases the secondary metabolites production (Herms and Mattson 1992, Strauss et al 2002, Stamp 2003).

With this study we aim to assess if and how it is possible to increase the internal levels of the major halogenated metabolites (bromoform and dibromoacetic acid) of *Asparagopsis taxiformis* in culture and how it affects the biomass growth. The effects of hydrogen peroxide (H_2O_2), of TAN and of CO_2 on the halogenated compound concentration were tested in the laboratory. Furthermore, the effects of TAN and CO_2 on the *A. taxiformis* halogenated metabolites content were investigated in the *Asparagopsis* spp.-integrated aquaculture system.

Materials and methods

Algae

Samples of the tetrasporophyte phase of *Asparagopsis taxiformis* were kindly provided from unialgal cultures at Stazione Zoologica, Naples, Italy (origin: Ischia, Naples, Italy). Seaweeds were maintained and grown as stock culture in a temperature and light controlled walk-in chamber (Aralab) at 15 ºC, 14:10 h L:D and 65 µmol photons m^{-2} s^{-1} in f/2 medium enriched natural seawater (Guillard and Ryther, 1962) which was exchanged on a weekly basis. The seaweeds were maintained at these conditions for more than one year.

H_2O_2 effects on VHC

Portions of 5 g FW of *A. taxiformis* from the stock cultures were incubated in each of 18 Erlenmeyers (1 L) containing enriched f/2 medium natural seawater. The seaweeds were kept at these condition during one 14:10 h L:D cycle. After this period, the culture mediums were replaced with natural seawater with 0, 0.1, 0.2, 0.5 and 2 mM of H_2O_2. Three replicates were done for each concentration level. The seaweed biomass was collected after 1 hour of exposure to H_2O_2. Three more vials enriched with 0.5 mM H_2O_2 were done to test for an exposure period of 3 hours. The incubation periods was based on literature data reporting that the VHC production rate is higher 1 to 2 hours after adding the H_2O_2 (e.g. Collen et al. 1994, Ohsawa et al. 2001). After the incubations, 0.2 g of biomass from each vial was preserved in liquid nitrogen for

analysis of iodoperoxidase (IPO) activity. Another biomass sample of 0.5 g was frozen at -20 ºC for VHC content analysis.

The effects of a H_2O_2 enriched medium over a longer period of time was tested as well. Portions of 10 g FW of *A. taxiformis* were incubated in two chemostats (1 L) during 8 days. The chemostats allowed a constant flow through supply of natural seawater enriched with f/2 medium. One chemostat was enriched with 0.1 mM H_2O_2. Portions of 0.2 and 0.5 g of biomass were taken at the days 1, 2, 3, 5 and 8 of the experiment and stored in liquid nitrogen for the IPO activity analysis and at -20 ºC for the VHC content analysis.

TAN and CO_2 effects on VHC

An orthogonal experimental was designed to investigate the interactive effects of C and N on both VHC content and biomass growth, with four treatments, –N/–C; –N/+C; +N/–C; +N/+C replicated twice. Five grams (FW) of *A. taxiformis* were inoculated in each of eight 1 L vials. Four of the vials were filled with natural seawater enriched with 100 µM TAN (–N), while in the other four vials the medium was enriched with 200 µM TAN (+N). Two vials of each N treatment were aerated with ambient air (–C), while in the other two vials of each N treatment the aeration was enriched with gaseous CO_2 (+C), during the light period. The medium was exchanged every day and the experiment ran for 8 days. In the –N treatment vials, the TAN concentration was depleted 8 hours after the medium renewal, while in the +N vials between 40 to 80 µM of TAN were still in the medium before its daily renewal (data not shown). The vials with ambient aeration kept the pH medium ~8.3 (~2.3 mM DIC) during the light period

(CO_2 limited for *A. taxiformis*), while in the CO_2 enriched vials (~2.9 mM DIC), the pH medium was maintained around 7 (non limiting CO_2 for *A. taxiformis*; chapter 5).

The experiment was done in a walk-in chamber (Aralab) at a temperature of 15 ºC and an irradiance of around 105 µmol photons m^{-2} s^{-1}. At the end of the experiment, the biomass was rinsed in paper to remove excess water and weighted to calculate weekly growth. Two portions of 0.5 g were taken and one was oven-dried (48 h; 60 ºC) for tissue C and N determination using an elemental analyser (Flash EA 1112, ThermoFinnigan), while the other was frozen at -20 ºC for VHC content analysis.

Effects of fish effluent flow rate on VHC

This experiment followed the same protocol described in chapter 5 for the outdoor tank cultivation experiments. In chapter 5 the experiment was designed to test the effects of a range of continuous flow rates of the fish farm effluent to the tanks (between 0.2 and 6 vol h^{-1}) and thus of associated TAN and DIC fluxes (water renewal rates x TAN and DIC concentrations) on the biomass growth. *A. taxiformis* biomass samples at the end of each experimental week between December and March were collected for VHC and C/N content analysis.

Iodoperoxidase activity tests

Iodoperoxidase activity was measured by following the conversion at 350 nm of I^- into I^-_3 (ε_M = 26,400 cm^{-1} M^{-1}), in the presence of H_2O_2 (Vilter et al. 1983). Algal samples (~0.2 g FW) were frozen in liquid nitrogen and grinded together with sterilized sand. 1 mL of 0.1M KH_2PO_4/K_2HPO_4 buffer (pH 6.2) was added and centrifuged for 5 min at

10000 rpm at 4 ºC to separate the supernatant from the solid part. The top phase (crude extract) was separated from the bottom phase and kept at 4 ºC.

The enzyme activity was determined indirectly by measuring the consumption of H_2O_2 at 25°C. Three assays were performed for each determination and their average used in the calculations (where 1 µmol/min of H_2O_2 consumed in the test was defined as 1U). The reaction solution contained 750 µL (0.13 M Na_2HPO_4 and 0.04 M citric acid) buffer (pH 6.2), 25 µL (0.2 M) KI and 25 µL crude extract. The reaction was started by adding 25 µL (26.6 mM) hydrogen peroxide. The reference contained only the buffer solution. Blank tests were measured where the crude extract was replaced by the buffer solution to determine oxidation of iodide by hydrogen peroxide (Vilter et al. 1983). To calculate the specific haloperoxidase activity, protein concentrations were measured using a method described by Bradford (1976).

VHC analysis

To quantify the major halogenated metabolites of *A. taxiformis*, samples were freeze-dried, weighed, extracted in methanol (MeOH), and analyzed by GC-µ-ECD using hexachlorobenzene as an internal standard. Fifty milligrams of freeze-dried algae were placed in a glass vessel and 1mL of MeOH was added. The vessel was immediately sealed and then sonicated for 15 minutes. Samples were left at -20ºC for 72 h to ensure esterification. The extract was filtered through a 0.45 µm Minisart RC15 syringe filter at room temperature. 100 µL of the filtrate were inserted in a glass vial and 100 µL of the internal standard methanolic solution was added at 15 ppm.

Gas chromatography was performed using an Agilent Technologies 6890N Gas Chromatograph equipped with a µ-ECD detector, automatic injection and a HP-5

column (30 m, 0.32 mm DI, 0.25 μm film thickness). The GC operation conditions were as follows: initial temperature 60ºC; hold 3 min; ramped at 10 ºC min^{-1} to 300 ºC, then held as this temperature for 1 min; carrier gas helium with a flow rate of 1,7 mL min^{-1}; injector temperature 310 ºC; detector temperature 300ºC; injection mode split 1/25. Commercial standards of bromoform and dibromoacetic (methyl ester) were used to identify and quantify the major peaks from methanol extracts of *A. taxiformis*.

Statistic analysis

One way ANOVA was used to analyse the effects of H_2O_2 concentrations on the IPO activity and VHCs content of *A. taxiformis*. Two way ANOVAs were used to analyse for significant effects of TAN and CO_2 concentrations on the biomass growth, and on the bromoform and DBA seaweed content treatments. No data transformations were necessary to satisfy the ANOVA assumptions of normality and heterogeneity of variance. Post-hoc comparisons were made using Tukey's test for multiple comparisons, when required. Linear regression analysis was used to assess the relationships of culture conditions, C/N content and biomass yield on VHC content. Significant effects were considered at $p<0.05$.

Results

H_2O_2 effects

The hydrogen peroxide had a significant effect on the IPO activity of *A. taxiformis* (ANOVA: $F_{5,17}$= 11.37, P<0.001; Fig. 1). Significant effects were observed at the highest concentration tested (2 mM H_2O_2), when IPO activity decreased to about one third of the control, and at 0.5 mM H_2O_2 after three hours incubation, when IPO activity decreased to about half of the control (Tukey's test P<0.05; Fig. 1). On the other hand, the H_2O_2 did not affect significantly the internal levels of bromoform (ANOVA: $F_{5,17}$= 1.99, P=0.153) and DBA (ANOVA: $F_{5,17}$= 2.13, P=0.132) (Fig. 1). Bromoform was the dominant metabolite in *A. taxiformis* with a content of 2.65±0.5 % DW, followed by DBA with 0.63±0.1 % DW.

The long-term exposure of *A. taxiformis* to 0.1 mM H_2O_2 revealed that IPO, bromoform and DBA decreased over time throughout the experiment (Fig. 2). The IPO activity decreased by 13 % after 24 hours, remaining at 70% of the control for the rest of the experiment. Only 48 hours after the beginning of the experiments decreases in VHC initial content (2.23 % DW of bromoform and 0.83 % DW of DBA) were observed (Fig. 2). At the eighth day, the levels of bromoform and DBA were respectively 26 % and 40 % of the control values.

TAN and CO_2 effects

The bromoform content of *A. taxiformis* was significantly affected by both TAN (ANOVA: $F_{1,4}$= 18.56, P=0.013) and CO_2 concentrations (ANOVA: $F_{1,4}$= 13.09,

P=0.022), whereas the DBA content was not affected (P>0.148). The lowest bromoform content of 1.75±0.09 % DW, observed under the +N/–C treatment, was the only significantly different value (Tukey's test P<0.05) (Fig. 3A). The other treatments showed similar levels of around 2.2 % DW). The biomass yield of *A. taxiformis* was affected as expected by TAN (ANOVA: $F_{1,4}$= 13.59, P=0.021) and CO_2 (ANOVA: $F_{1,4}$= 22.8, P=0.009) concentrations. The lowest biomass growth values (0.33±0.22 g wk^{-1}) were measured under C and N limitation (–N/–C, Fig. 3). Enriching the culture medium with CO_2, but maintaining the same TAN levels, the biomass yield increased significantly to 0.95±0.13 g wk^{-1}. These values were similar to the ones measured under C limitation but not N (+N/–C). The seaweeds growing in the medium enriched with both C and N presented the highest biomass growth (1.3±0.1 g wk^{-1}).

No significant effects of *A. taxiformis* biomass growth on either bromoform (R^2=0.08, P=0.843) or DBA (R^2=0.11, P=0.794) content were found (Fig. 4). On the other hand, both bromoform and DBA content always increased in response to increased C availability (dashed slopes, Fig. 4) whereas it always decreased in response to increased N availability (solid slopes, Fig. 4). This effect was more pronounced in the case of bromoform. As a result of this, both bromoform and DBA contents increased with the C/N ratio of *A. taxiformis* (bromoform: R^2=0.84, P<0.005; DBA: R^2=0.81, P<0.005; Fig. 5).

Fish effluent flow rate effects

Under mass cultivation conditions of *A. taxiformis*, the water renewal rates had a significant, positive, linear effect on the species bromoform content (R^2=0.65, P<0.001), but not on the DBA content (R^2=0.2, P=0.36) (Fig. 6). The water renewal effect reflects

the combined effects of the available TAN and DIC to the cultures, which are directly associated to water flux. As these influence biomass growth, this variable was positively related to the bromoform content (Fig. 7). The pH values recorded in the tanks were inversely related with the water renewal (see chapter 5), and consequently to the bromoform content (R^2=0.73, $p<0.001$) (Fig. 8).

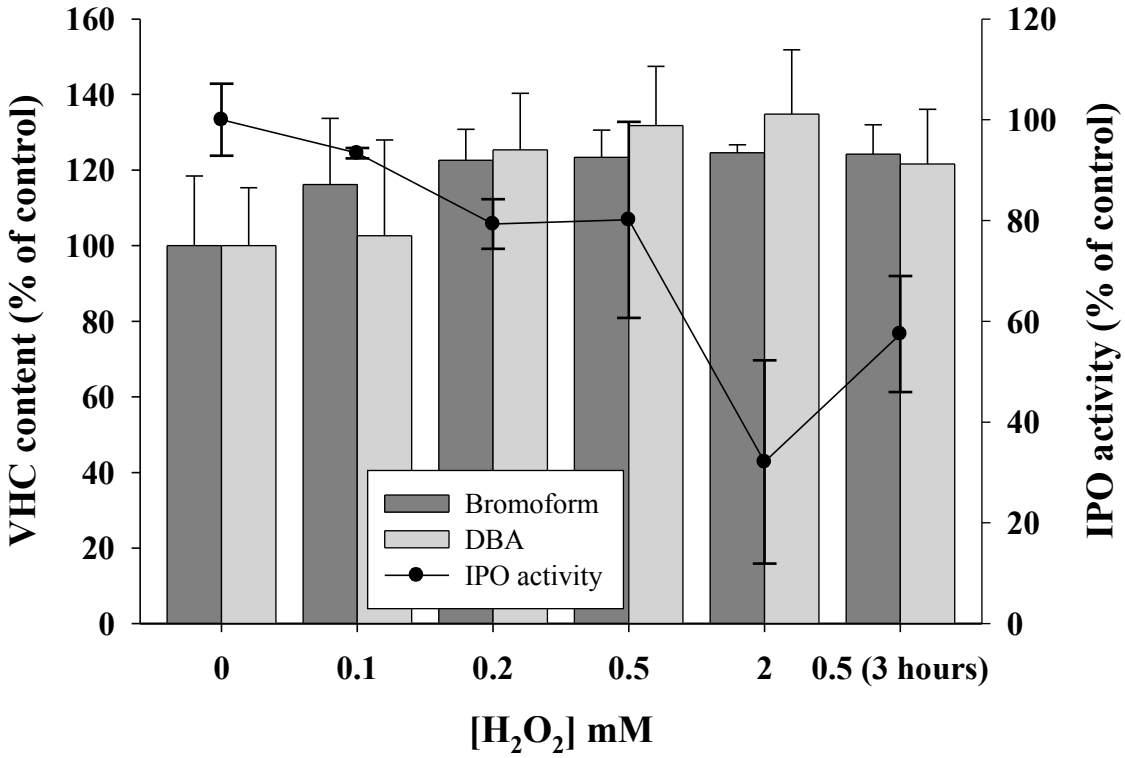

Fig. 1 – Percentage of the iodoperoxidase (IPO) activity and the internal levels of bromoform and DBA in *Asparagopsis taxiformis* incubated during one or three hours (when indicated) to several H_2O_2 concentrations, in relation to the control values (no H_2O_2 addition), n=3.

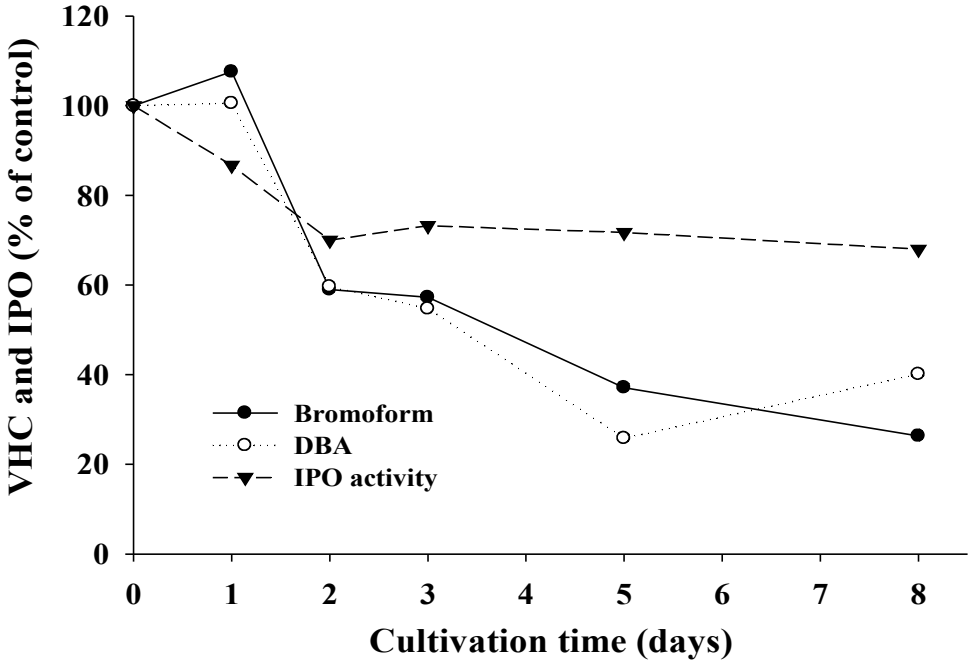

Fig. 2 – The evolution with time of percentage of the internal bromoform and DBA levels and the iodoperoxidase (IPO) activity in *Asparagopsis taxiformis* incubated at 0.1 mM H_2O_2, in relation to the control values (no H_2O_2 addition).

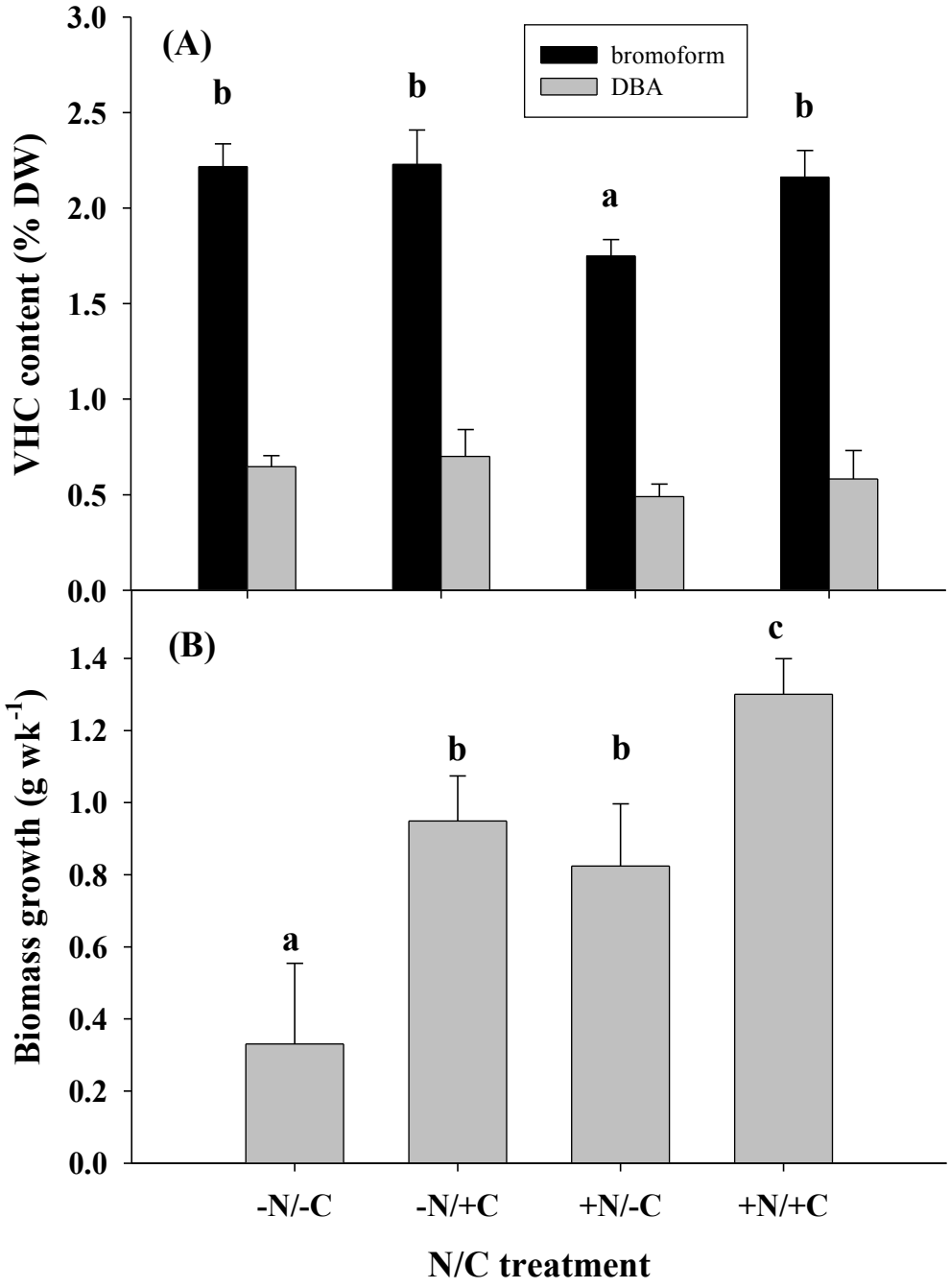

Fig. 3 – (A) Internal levels of the two major halogenated metabolites in *Asparagopsis taxiformis* incubated at four N/C treatments during one week and (B) respective biomass yield, n=2. Treatments with different letters differ at P=0.05 (Tukey's multiple comparison).

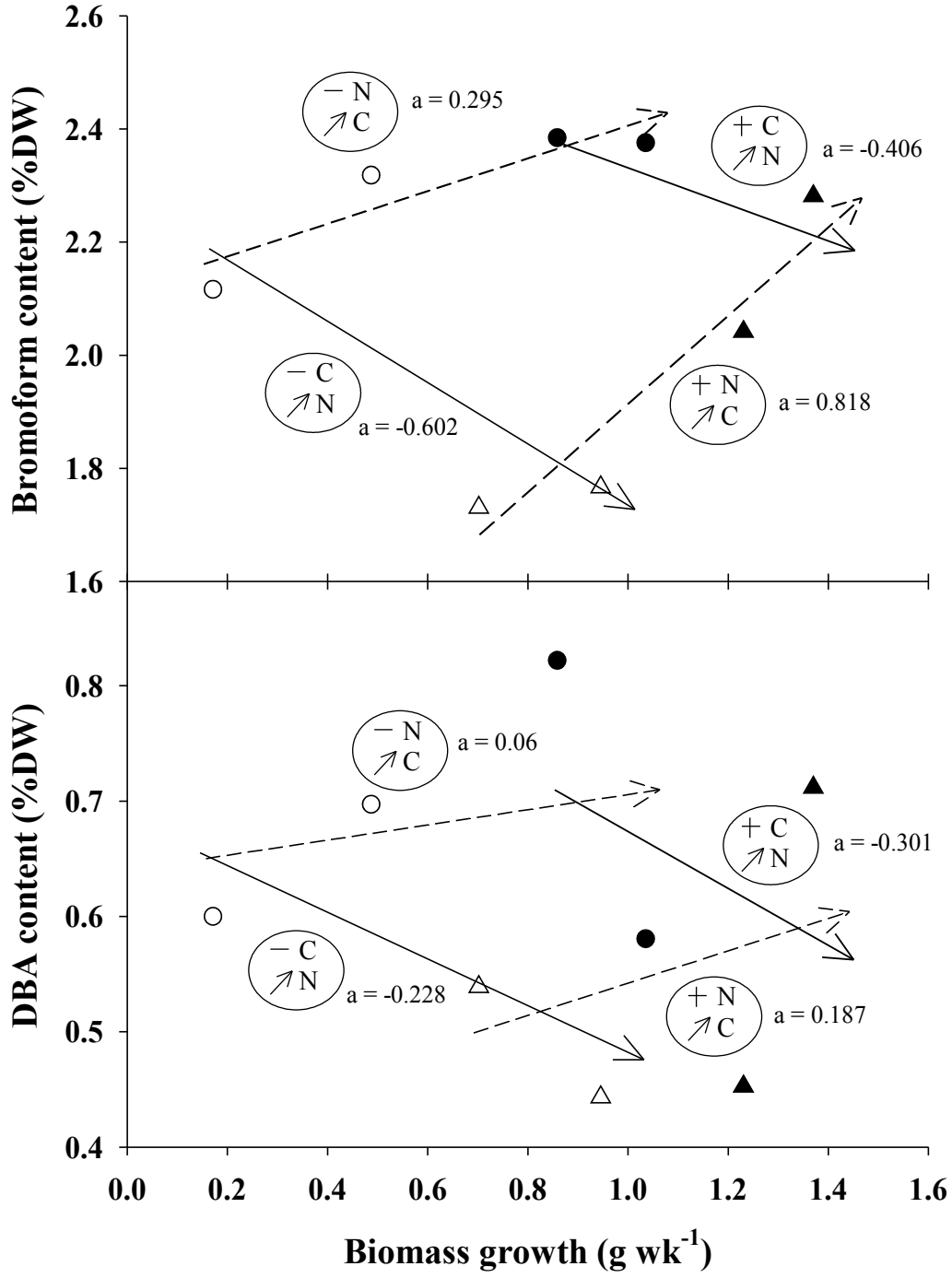

Fig. 4 – Biomass yield vs. the internal levels of the two major halogenated metabolites in *Asparagopsis taxiformis* incubated in four N/C treatments during one week. Circles correspond to -N treatments, while triangles to +N treatments. Open symbols correspond to -C treatments, while filled symbols to +C. The solid linear regression line is the relation of the metabolites with the different levels of TAN, whereas dashed linear regression line considers only the CO_2 effects (a = slopes of the linear regressions).

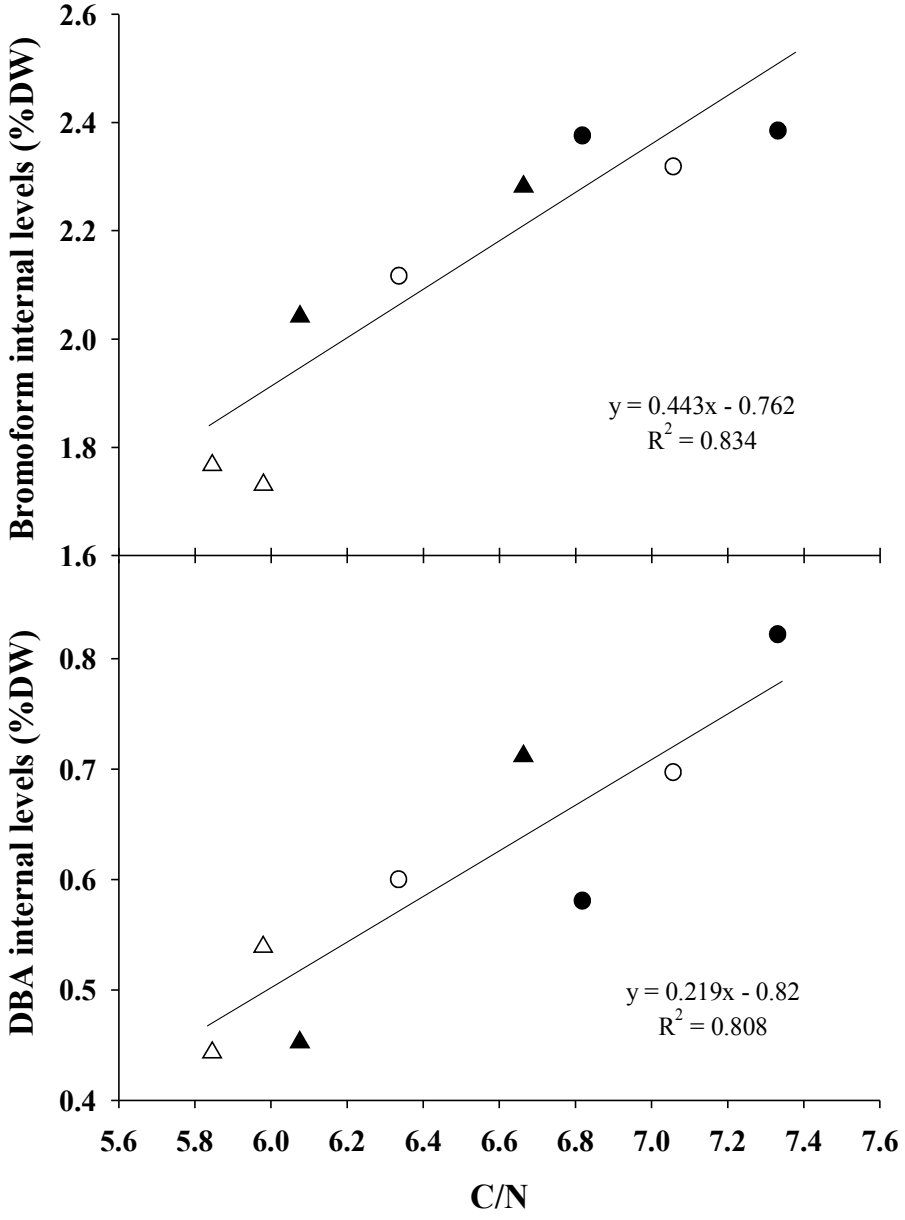

Fig. 5 – C/N ratio in the biomass vs. the internal levels of the two major halogenated metabolites in *Asparagopsis taxiformis* incubated in four N/C treatments during one week. Circles correspond to -N treatments, while triangles to +N treatments. Open symbols correspond to -C treatments, while filled symbols to +C.

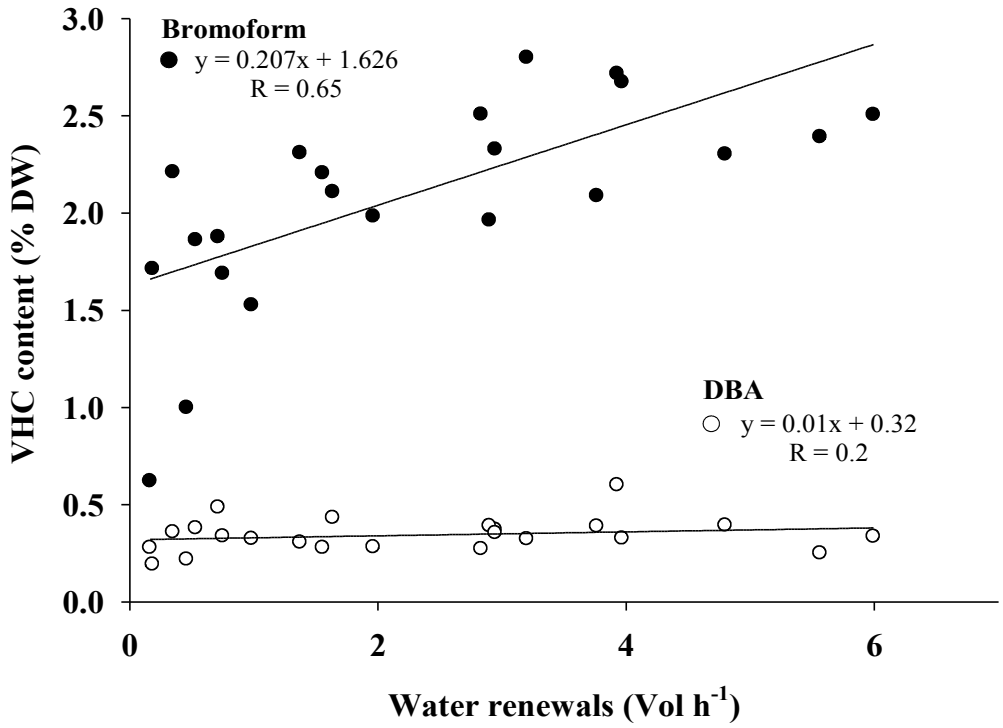

Fig. 6 – Bromoform (filled symbols) and DBA (open symbols) internal levels in *Asparagopsis taxiformis* cultivated in tanks with fish effluents supplied at different water renewal rates.

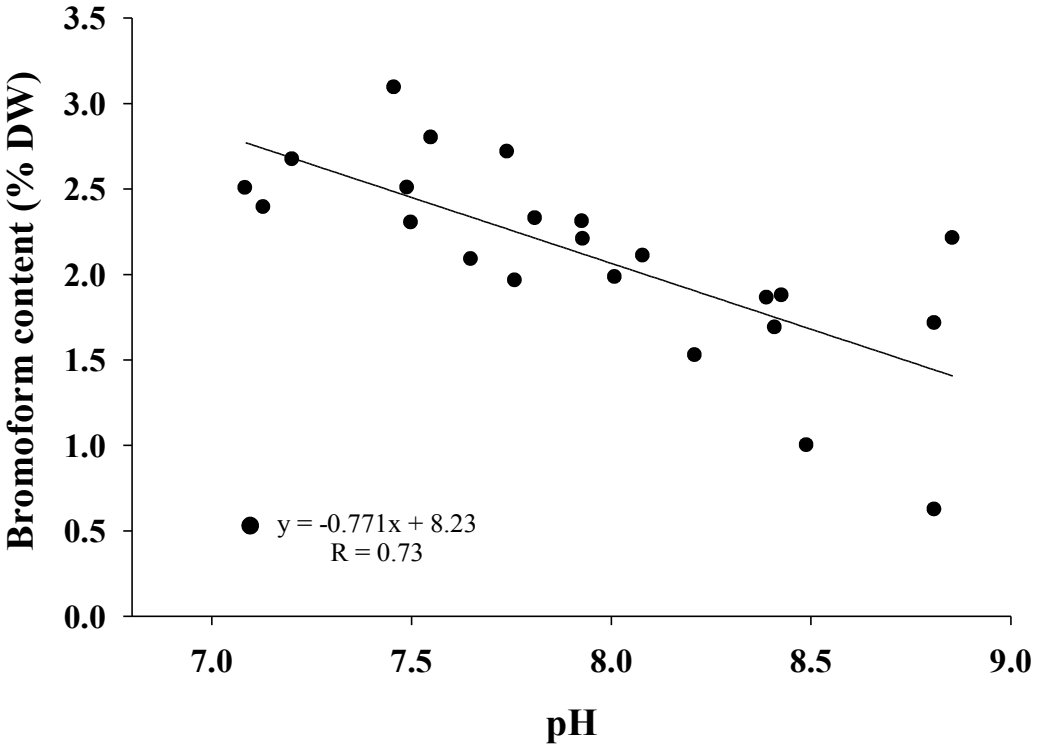

Fig 7 – Bromoform internal levels in *Asparagopsis taxiformis* cultivated at different water renewal rates and their relation with the culture medium pH values at solar midday.

Figure 8

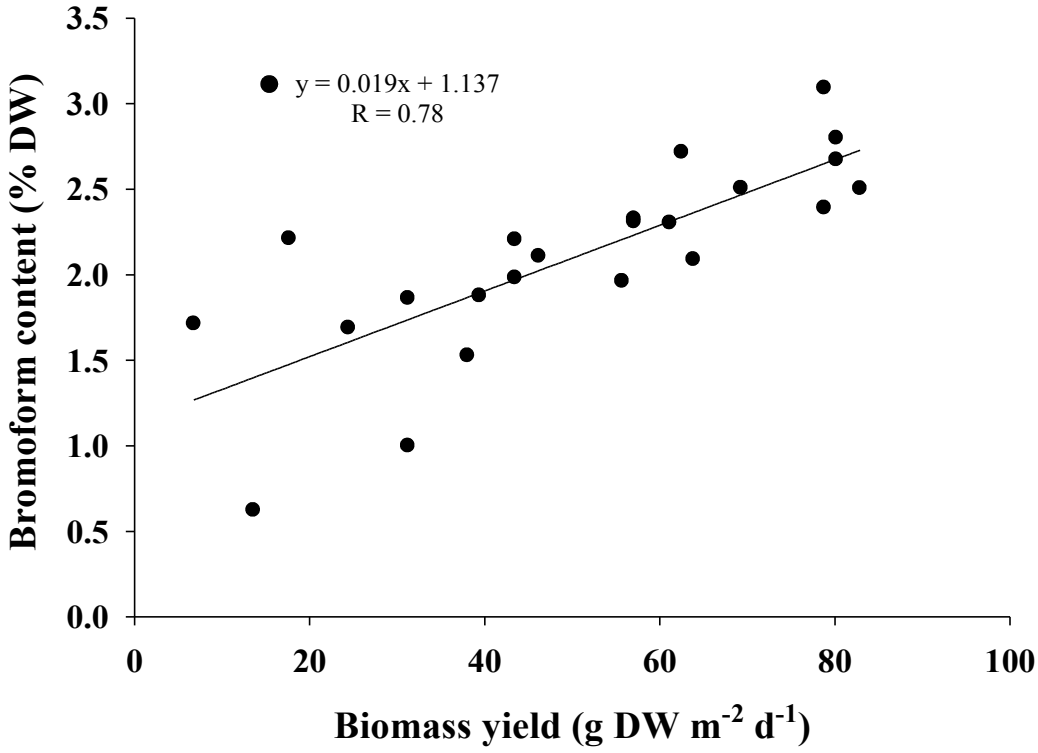

Fig. 8 – The relationship of the *Asparagopsis taxiformis* yield and respective internal levels of bromoform in biomass cultivated at different water renewal rates in the integrated cultivation system.

Discussion

This study showed that the availability of TAN and CO_2 influenced the bromoform content of *A. taxiformis* tissue. Contrary to what was expected, the addition of H_2O_2 to the medium resulted in a decrease of the haloperoxidase activity with a consequent decrease of the both bromoform and DBA. The inhibition of the enzyme activity by H_2O_2 enrichment has been seldom reported in algae. This response may be related to the enzyme prosthetic group. Two distinct types of marine haloperoxidases have been identified: those that contain Fe-heme as a cofactor and those containing vanadium (V) (Buttler and Walker, 1993). Red and brown algae tend to contain vanadium as the cofactor (Neidleman & Geigert 1986; Wever & Krenn 1990; Butler 1998), while in green algae and diatoms both types have been reported (Wever & Krenn 1990; Moore et al. 1996; Verdel et al. 2000). V-bromoperoxidase activity is enhanced when H_2O_2 is added to the medium at relatively high concentrations (Collén et al. 1994, Sundström et al. 1996, Pedersén et al. 1996, Manley & Barbero 2001, Ohsawa et al. 2001). For example in the red alga *Corallina pilulifera*, the optimum H_2O_2 concentration for the enzyme activity ranged between 0.5 and 5 mM, while at higher concentrations inhibition occurred (Itoh et al. 1986). Heme-containing peroxidises, on the other hand, are reported to be more sensitive to hydrogen peroxide concentration (Zuurbier et al. 1990). In the red microalga *Porphyridium purpureum*, the iodoperoxidase activity was inhibited at H_2O_2 concentrations above 0.1 mM. This and other evidences led the authors to conclude that *P. purpureum* has a heme containing haloperoxidase (Murphy et al. 2000).

Although rare in red macroalgae, Fe-heme containing haloperoxidases were already suggested for the red species *Cystoclonium purpureum* (Murphy and O'hEocha, 1973) and *Bonnemaisonia hamifera* (McElvany et al 1979), this one belonging to the same family of *A.*

taxiformis (Bonnemaisoniaceae). This order of evidences indicates that *A. taxiformis* contains heme-haloperoxidases, but only a proper characterization of the enzyme will allow the identification of its prosthetic group. Considering the applied objective of improving the economic value of *A. taxiformis* biomass, we conclude that adding hydrogen peroxide to the culture mediums does not increase the bromoform and DBA tissue contents. Consequently, abiotic stress such as high light conditions, which induces the internal production of H_2O_2 by the algae, should be avoided.

Whereas the availability of CO_2 resulted in higher bromoform content of *A. taxiformis* tissue, the availability of TAN had the opposite effect. Under integrated aquaculture, the supply of higher water renewals provides higher TAN and CO_2 availability to the seaweeds, which resulted in an increase of the bromoform content of *A. taxiformis*. This is an indication that CO_2 availability is more important for bromoform production in *A. taxiformis* than the TAN availability, at least at CO_2 limiting levels for photosynthesis. This type of response to CO_2 availability relates well with the described importance of CO_2 for *Asparagopsis* spp. photosynthesis and growth (chapter 5 and 6). *A. taxiformis* photosynthetic rates are only fully saturated at pH values lower than 7.7. From pH 8 to pH 9 (where CO_2 is virtually absent) the species photosynthesis and growth becomes severely limited (chapter 6) and a steep decrease of bromoform occurs (this chapter). These results support the Bryant et al. (1983) carbon-nutient balance hypothesis, which states that CO_2 limiting conditions for photosynthesis may affect non–structural carbohydrate pools, decreasing both growth and production of carbon based secondary compounds. It was observed that the tissue C/N ratio and growth of cultivated *A. taxiformis* were positively related to bromoform production (no growth costs associated with bromoform production).

No seasonal variation trend in the bromoform content of cultivated *A. taxiformis* was observed. Little is known about the effects of environmental factors on the production of

volatile halocarbons by algae. Abrahamsson et al. (2003) studied the influence of temperature on volatile halocarbons release by several species. They concluded that the production of certain halocarbons increase with temperature in certain algal species but found no response patterns to temperature change that were consistent for all species tested or for all halocarbons studied. The bromoform content of *A.taxiformis* did not increase with temperature, at least from December to March.

Our study shows that the CNB hypothesis provides a theoretical template for predicting how nutrient levels will affect the major VHC, especially bromoform content in cultivated *A. taxiformis*. The integrated aquaculture of Bonnemaisoniaceae using fish pond effluents is therefore an ideal system to produce high amounts of biomass with high contents of economic valuable halogenated compounds. The metabolites content can probably be further maximized by supplying non limiting CO_2 rates for photosynthesis, along with limiting TAN conditions, i.e. when the C/N ratio is maximized. In integrated fish/seaweed aquaculture, this nutrient balance can only be attained using low water renewal rates and the addition of an extra carbon source to the cultures, which will increase the costs of biomass production.

CHAPTER 8

General Discussion

The suitability of the tetrasporophytes of Bonnemaisoniaceae for inland integrated aquaculture, particularly those of the genus Asparagopsis, was demonstrated. The species biofiltration and biomass production performances are the highest reported, exceeding those of the commonly used *Ulva* spp. biofilters. Furthermore, the members of this family have high economic value resulting from the biological activity of their halogenated metabolites. By investigating the physiological characteristics of the species, the cultivation conditions (sunlight, nitrogen and carbon) that maximize biofiltration, biomass production and the internal levels of the two major halogenated metabolites (bromoform and dibromoacetic acid) were established.

Biomass density (light availability)

One major parameter to manipulate in algae cultivation systems is the biomass stocking density. It influences the light availability inside the tanks and thus the biomass production and the development of nuisance species (Bidwell et al. 1985, Grobbelaar et al. 1990). The establishment of the ideal biomass density depends on the light environment

inside the tanks, the species light requirements and the individuals' circulation pattern in the tanks (light:dark frequencies). The stocking density that maximized the weekly production of *A. armata* was found to be 5 g FW L^{-1} (2.4 kg m^{-2}). In one week, the culture densities increased to 8 g FW L^{-1} and to 10 g FW L^{-1}, depending on the season. At this range of densities, the individuals spend the majority of their time in virtual darkness, receiving light only in the first few centimetres of the tanks (1/10 of their time) and a short flash of intense light at the surface (Mata et al. 2006 - chapter 3). Consequently, the species photosynthetic system must be well adapted to low light environments. *A. armata* P-I curve patterns indeed followed a typical response of shade adapted seaweeds, with a high photosynthetic efficiency at low light levels and some degree of photoinhibition at high light levels.

In tanks with biomass densities of 5 g FW L^{-1} or higher, the photosynthetic performance of the individuals depends mostly on the light-limited portion of the P/I curve. *A. armata* photosynthesis was revealed to be highly efficient. The exposure time of the circulating thalli to saturating levels of light is probably too short at these densities for photoinhibition to occur. Photoinhibition was only observed in individuals cultivated at densities lower than 5 g FW L^{-1} (higher light levels in the tanks) at the solar midday. On the other hand, photoinhibition was shown to be dynamic (no photodamage of PS II reaction centres) and the potential quantum yield (Fv/Fm) of the PS II recovered along the day with decreasing light intensities. Recovery of the PS II was also observed in individuals transferred to shade after one-hour exposed to full solar irradiance, a response characteristic of sun adapted algae (Häder et al. 1996b, Jiménez et al. 1998). This type of photosynthetic plasticity is an essential characteristic of a seaweed species for tank cultivation.

The determination of the optimum cultivation density must also take into consideration the development of epiphytes or nuisance species in the cultures, a common problem in red seaweed aquaculture (Bidwell et al. 1985, Friedlander 1992, Demetropoulos

and Langdon 2004). The mass cultivations of *A. armata* and *A. taxiformis* kept free from nuisance species during most of their cultivation periods. The low light levels registered inside the tanks may limit the development of green opportunistic species, probably not so efficient in using low light levels. The exudation of halogenated metabolites by *A. armata* to the surrounding culture medium (Paul et al. 2006a) may contribute to deter other species to develop, even though in these flow through culture systems the medium is quickly renewed. The completely absence of epiphytes on the thalli surface of *Asparagopsis* spp. is related with the surface-active antimicrobial agents, which limits their growth (Paul et al 2006a).

Rates of effluent supply (N and C availability)

Once the ideal inoculating biomass density was established, the objective was to make efficiently use of total ammonia nitrogen (TAN) and dissolved inorganic carbon (DIC) present in the fish effluents, by manipulating the water renewals to the seaweed tanks. The biomass yield and TAN uptake of *Asparagopsis* spp. increased asymptotically with the amount of nutrients supplied to the tanks. In this integrated cultivation system, effluents supplied at a rate of ~3 vol h^{-1} provided the quantity of nutrients that maximizes the TAN removal capacity and the biomass yield. This optimal effluent renewal rate was fairly constant along the seasons and in different years.

The performance of seaweed species in integrated aquaculture has always been related exclusively to nitrogen availability (see the review by Neori et al. 2004). However, other factors than nitrogen may play an important role as indicated by the observations that the water renewal rate at which the maximum biomass yield of *Asparagopsis* spp. was achieved (~3 vol h^{-1}) was relatively constant throughout the year even though the

correspondent TAN fluxes (water renewal rates x TAN-concentrations) varied considerably. For the first time, the DIC quantity and forms in the water at all stages of an integrated aquaculture system were characterized. This allowed the evaluation of the DIC contribution by the fish unit and of the DIC usage by the seaweeds and thus the assessement of its importance to the *Asparagopsis* spp. production. The species' mechanisms of carbon use, reported for the first time, were also investigated. Both *Asparagopsis* species showed to rely mainly on free CO_2 to meet their photosynthetic needs, lacking an efficient mechanism to use HCO_3^- from the water. Only pH levels below 8 therefore provide enough CO_2 to maximize the photosynthetic rates of the species. *Asparagopsis* spp. were not able to use carbon for photosynthesis at medium pH values above 9, where the dissolved CO_2 is virtually absent and the uncatalyzed HCO3- dehydration rate is very slow to account for the photosynthetic requirements (Cook et al. 1988, Maberly 1990, Johnston et al. 1992, Choo et al. 2002).

In this integrated fish/seaweed aquaculture, CO_2 respired by the fish lowered normal seawater pH (around 8.2) to values around 7. The photosynthetic uptake of CO_2 by the seaweeds results in a near stoichiometric production of hydroxyl ions, which increases the pH (Cook et al. 1988, Prins and Elzenga 1989, Raven 1997). In tanks with such a high seaweed biomass density, and especially at the highest photosynthetic period of the day, a rapid depletion of CO_2 occurs with the consequent increase of the pH medium up to 9 when no free CO_2 is available. Increasing the seaweed tanks' water renewal rates increases the supply of CO_2 to the seaweed tanks. Saturating CO_2 levels to *Asparagopsis* spp. photosynthesis (culture medium pH values below 8 at all times) were only attained in the tanks supplied with ~3 vol of effluents h^{-1}. Consequently the seaweeds attained maximum rates of both carbon removal and biomass growth in these tanks.

The production of species that depend almost exclusively on dissolved CO_2 for photosynthesis may be more susceptible to C than to N availability (see Rivers and Peckol, 1995). Evidence of the importance of DIC flux on the *A. taxiformis* production was revealed by the multiple linear regression model of biomass yield (Chapter 6), which revealed that the *A. taxiformis* yield was more correlated to DIC fluxes than to any other abiotic parameter (temperature, irradiance and TAN). Laboratory experiments (Chapter 7) further confirmed that CO_2 had a stronger influence on biomass yield than the TAN concentration.

Biomass valorisation

The CO_2 availability to *A. taxiformis* was also more important for the production of the major halogenated metabolite (bromoform) than the TAN fluxes (chapter 7). Whereas the availability of CO_2 resulted in higher bromoform content of *A. taxiformis* tissue, the availability of TAN had the opposite effect. Under integrated aquaculture, the supply of higher water renewals provides higher TAN and CO_2 availability) to the seaweeds, which resulted in an increase of the bromoform content of *A. taxiformis*. These results support the Bryant et al. (1983) carbon-nutient balance hypothesis, which states that CO_2 limiting conditions for photosynthesis may affect non–structural carbohydrate pools, decreasing both growth and production of carbon based secondary compounds that deter herbivores. It was observed that the tissue C/N ratio and growth of cultivated *A. taxiformis* were positively related to bromoform production. The integrated aquaculture of Bonnemaisoniaceae using fish pond effluents is therefore an ideal system to produce biomass with high contents of economic valuable halogenated compounds.

Contrary to the expected, the external addition of hydrogen peroxide, a substrate involved in the bromoform production (e.g., Ohsawa et al. 2001), in the cultures did not increase the internal levels of both bromoform and DBA in *A. taxiformis* tissue. On the contrary, it inhibited haloperoxidase activity with a consequent decrease of the two VHCs internal levels. This response may be related to the enzyme prosthetic group. Contrary to most of the red alga haloperoxidases that have vanadium as a cofactor, *A. taxiformis* may contain a heme-haloperoxidase, which is inhibited by high hydrogen peroxide concentrations (Zuurbier et al. 1990).

Performance of the *Asparagopsis* spp. biofilter/production units

When *A. armata* is cultivated at nutrient (including nitrogen and carbon) saturation conditions (above 3 vol h^{-1}), each square meter of cultivation unit removes between 2.8 g N day^{-1} in winter and 8.8 g N day^{-1} in late spring from the fish effluents, producing between 40 g DW day^{-1} and 120 g DW day^{-1} of biomass. The *A. taxiformis* yield and biofiltration performance was in general slightly lower than the *A. armata* but followed a similar seasonal pattern (Chapter 6). These biofiltration and growth values were significantly higher than other seaweed biofilters in integrated aquaculture systems (Schuenhoff et al. 2006 - chapter 2). As well, they were higher than the fast growing *Ulva rigida* when compared directly in this system under the same conditions (Chapter 4).

The higher performance of a filamentous form species over a sheet-like species would not be expected according to the functional-form model proposed by Littler et al. (1983). However, the specific light regime in the tanks may present different advantages to the different morphologies. The round-shape, “pom-pom” type morphology of *Asparagopsis*

spp. tetrasporophytes rollover constantly in the aerated cultures, which increases the light/dark frequency conditions to the cells, in comparison with the blade type morphology of *Ulva* spp. Grobbelaar et al. (1996) revealed that seaweed productivity, and in particular that of low light acclimated algae, can be enhanced between 1.68 and 6 times with this type of light regime. *Asparagopsis* spp. in the dense culture tanks are low light acclimated (Mata et al. 2006 - Chapter 3).

Biofiltration and biomass yield per surface area obtained for *Ulva rigida* in this cultivation system were the highest ever reported for *Ulva* spp. cultivated in integrated fish/seaweed aquaculture (Chapter 4). The remarkable performances obtained for both genera in this system may probably be related with the characteristics of the cultivation system itself. The tanks in this system have a smaller volume and the wall is translucent, allowing the penetration of about 70% of the incident PFD. This increases the light exposure surface / culture volume compared with tanks used elsewhere, resulting in a higher light use efficiency and consequently higher performance per surface area. However, the use of these small highly productive tanks may not be sustainable when considering a large-scale production unit. They require high man-labour per surface area for the regular maintenance and operation performances, such as the harvest of the biomass and especially the tank wall cleaning to prevent the development of unwanted species. Lower performances should thus be expected when using higher volume tanks with lower ratios of light exposure surface / culture volume. An important aspect to pay attention to in the engineering of such larger production tanks is related with the proper scaling and positioning of the water outflow screens. Small volume tanks have the advantage of allowing the renewal of higher water volumes and thus the supply of higher nutrient and carbon to the seaweeds. This aspect is particularly important to exploit the maximum performance of *Asparagopsis* spp. cultivation, which depends on relatively high water renewal rates (at least 3 vol h^{-1}). On the

other hand, the production of species with mechanism to use HCO_3^- as an alternative source of carbon for photosynthesis, such as *Ulva* spp., may not be so susceptible to high water turnover rates. In this cultivation system, the biomass yield of *U. rigida* decreased by ~35% from the highest water renewal tank (pH 8) to the lowest one (pH 10), whereas the biomass production of *A. armata* decreased ~75%.

An advantage of using higher volume tanks for the seaweeds is that the water temperature is kept more stable along the day. Inside the small tanks it oscillated around 6 ºC along the day during early summer. The water temperature above 27°C, reached in June, was lethal for *A. armata* (Schuenhoff et al. 2006 - Chapter 2). A. *taxiformis* persisted longer in cultivation but its production crashed when water temperatures rose above 29 ºC (Chapter 6). This was still below the 31 ºC described as the lethal limit temperature of this species (Ní Chualáin et al. 2004) observed in pure cultures under constant laboratory conditions. These temperature limitations render the year round cultivation of both *Asparagopsis* species impossible in southern Portugal.

To overcome this problem, the co-cultivation of both species, *A. taxiformis* and *U. rigida*, may be considered. During the 9 months period of *A. taxiformis* cultivation, each square meter of its tanks would produce an average of 24 kg DW of biomass. This biomass production would result in a removal from the fish effluents of 1.52 kg of N and 8.23 kg of C in the form of NH_4^+ and CO_2 respectively. During the remaining three months, each square meter of *U. rigida* would produce about 6 kg DW of biomass and remove 0.21 kg of N and 2.1 kg of C from the effluents. Should future legislation pressure the aquaculture industry to internalize environmental costs for their waste discharge, N and C biofiltration may turn from just a by-product of seaweed production, with all the inherent environmental benefits, into an economic benefit to the producer.

The cultivation of more than one species may bring potential benefits to face eventual problems with one of the species production or species market value fluctuations. It is therefore important to explore other market applications for the species. *Ulva* biomass is still sub-valorised, but is now gaining interest as a potential source of cell wall polysaccharides (especially ulvan), whose physicochemical and biological properties make them attractive candidates for novel functional and biologically active polymers for the food/feed, pharmaceutical, chemical aquaculture, and agriculture domains (Lahaye and Robic 2007). Apart from the cosmetic market, the properties of the VHCs of *Asparagopsis* spp. can also be explored for other applications including agriculture (biopesticides), painting industry (antifouling agents), food industry (preservatives). An aspect to explore in both research and practical application is the impact of the seaweeds bioactive compounds on the microbiological properties of the water connecting algae and animals in polyculture recirculation systems, especially the impact on fish pathogenic bacteria (Pang et al. 2006). *Asparagopsis armata* extracts showed to be highly effective against fish pathogenic bacteria *in vitro* (Bansemir et al. 2006). However, studies linking waterborne seaweed metabolites with demonstrable antimicrobial properties are rare. This has been recently done with two species of algae, *Delisea pulchra* and *Asparagopsis armata* both members of the family Bonnemaisoneaceae (de Nys et al. 2006, Paul et al. 2006a).

Asparagopsis spp. proved to have the characteristics to be considered as a seaweed biofilter of inland mariculture practices. Another characteristic not highlighted before but with extreme importance for mass seaweed cultivation, is that tetrasporogenesis never occurred during the whole cultivation periods. The search for novel seaweed species with similar characteristics as those of *Asparagopsis* spp. should continue to successfully integrate seaweeds in the animal aquaculture industry. The presence of special structures in the cells that store metabolites could be a useful directive tool to select species with novel,

active metabolites. Filamentous algae, in particular those of the Ceramiaceae may be a promising source of new compounds (Paul 2006).

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