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Effect of Light Quality and Nitrogen Availability on the Biomass Production and Pigment Content of *Palmaria palmata* (Rhodophyta)

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Macroalgae constitute a huge underexploited resource for compounds of interest to the health industry. External factors strongly influence the concentration of these compounds in the algal tissue. This implies a potential for optimizing the growth conditions for cultivated macro algae in order to promote production of valuable compounds. The objective of this study was to determine the influence of light spectral composition (red, blue, and white) and nutrient treatment (30 and 440 µM NO_3^-) on chlorophyll a and carotenoid concentration as well as growth rate of the red algae *Palmaria palmata*. The results show that the nitrogen load has a larger effect on the pigment concentration and growth rate in the algae, than the light treatments. Experiments with different light spectral composition showed the highest pigment content with white and blue light while red light was less effective. In addition, samples cultivated under white light had higher growth rates compared to red and blue light. Therefore, to increase the concentration of nutritionally valuable pigments cultivation strategies in marine production should be to use blue or white light and use high NO_3^- concentrations.

1. Introduction

Global growth in population has increased the pressure on traditional sources for drugs and food. This steadily increasing demand for new drugs and food compounds based on natural products has led to a need to utilize biomass from alternative sources. The marine environment, which includes huge diversity of organisms with specific biological properties, is one of the most underutilized resources and its industry provides an estimated total annual value of US$ 5.5 - 6 billion (McHugh, 2003). In the last decades, high amount of nutrients and functional compounds such as proteins, healthy fats, pigments, sugars, fibers etc. have been discovered in different species of marine macro- and microalgae. Almost all genera of seaweeds have shown some bioactive effects such as antioxidant, antibacterial, anti-fungal, anti-viral and anti-inflammatory. Furthermore, using seaweeds as feed supplements in aquaculture and also for livestock have shown beneficial effects on their health and products (Holdt and Kraan, 2011). This indicates great potential of macroalgae as functional food ingredients or for the extraction of valuable compounds.
During the last 10 y the production of wild seaweed did not change considerably whereas cultivation of seaweeds has been increasing. Although, production of wild and cultured seaweed has reached almost $18.2 \cdot 10^6$ t in 2010, it is still not sufficient to fulfill the growing demand (FAO, 2012). Therefore, continuous efforts have been made to study the life cycle and eco-physiology of already cultivated or promising macroalgae species. Various environmental parameters such as light (quantity as well as spectral composition), salinity, temperature, and availability of nutrient can influence chemical quality (e.g. content of secondary metabolites) of macroalgae (Aguiliera et al, 2002; Sagert and Schubert, 1995a).
Among several edible red seaweeds, *Palmaria palmata* (dulse) is a relatively common Atlantic seaweed being harvested and produced for human consumption, cosmetics, and feed in aquaculture. A high content

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of protein was recorded for *P. palmata* (35 % of dry weight) which is comparable with contents found in protein rich legumes such as soybeans. In addition to the relatively high protein content, *P. palmata* also contains valuable pigments, such the carotenoids β-carotene and lutein (Morten et al., 1980). Carotenoids are known for their anti-cancer, anti-oxidant properties and activity against obesity which makes them an attractive class of high-value compounds. The worldwide carotenoid market is estimated to be U$ $786 \cdot 10^6$ million in 1999 with an estimated average annual growth rate of 2.9 %. Recently, lutein's global market earned revenues of US$ $105 \cdot 10^6$ in 2006 and it is estimated to reach nearly US$ $125 \cdot 10^6$ by 2013 (Holdt and Kraan, 2011). Moreover, the worldwide demand for β-carotene has specifically risen from ca. US$ $237 \cdot 10^6$ in 2004 to ca. US$ $248 \cdot 10^6$ in 2009. The function of carotenoids in living algae is primarily to act as antioxidants, but also to act as accessory pigments to chlorophyll a, absorbing incoming light either coupled to photosynthesis or as photo-protection (Raven et al, 2005). Carotenoids concentration is thus regulated by various parameters primarily light intensity (Sagert and Schubert, 1995b) and light quality (Sagert and Schubert, 1995a), but also by the amount of nutrient (e.g. nitrogen) available (Kopsell et al, 2007). This opens for the possibility of implementing manipulation of environmental factors in macroalgae cultivation practices with focus on improved content of high value compounds.
In the present work, the effect of light spectral composition and nitrogen availability on the biomass yield and production of photosynthetic pigments such as carotenoids and chlorophyll in *P. palmata* is investigated in an experimental laboratory set-up.

2. Materials and Methods

2.1 Algal growth experiment

2.1.1 Sample collection

Healthy fronds of the red algae *Palmaria palmata* were collected on 17th April 2012 from a natural population at a depth of 6 meters at Fornæs, Denmark (N:56°26'46'' and E:10°58'09''). The fronds were kept in natural seawater with a salinity of 28 psu and low nutrient content (< 10 µM Dissolved Inorganic Nitrogen (DIN)) in a climate room (white light (approximately 55 µmol photons $s^{-1}m^{-2}$), a photoperiod of 16:8 h (light:dark), and a temperature of 10 °C).

2.1.2 Experimental set-up

Prior to the experiment, the *P. palmata* fronds were acclimatized for 7 days at white light (approximately 55 µmol photons m^{-2} s^{-1}) and two different nutrient treatments: a low nutrient treatment (30 µM NO_3^-) and a high nutrient treatment (440 µM NO_3^-). Standard f/2 nitrate, phosphate, trace metal and vitamin stock solutions were used as nutrient supply (Guillard and Ryther, 1962). At the beginning of the experiment, 1.5 L of artificial seawater (Coral Pro Salt, Red Sea) mixed to a salinity of 25 psu was added to 18 glass beakers each of 2 L volume. Two different nutrient treatments were prepared: a low nutrient treatment (30 µM nitrate) and a high nutrient treatment (440 µM of nitrate). In each nutrient treatment three beakers were exposed to one of three different light qualities (red (RL), white (WL) or blue (BL)) of the approximately same intensity (approximately 55 µmol photons s^{-1} m^{-2}). This experimental design gives three replicates of every combination of nutrient and light treatment. The light was supplied by LED lamps (Pack Lamp RBG, RS Components, Ltd. UK). *P. palmata* biomass was added to every glass beaker (approximately 15 g fresh weight (FW) per beaker). Every frond was split between several beakers in order to minimize the variation. Light was measured regularly throughout the experimental period. The experiment was conducted in a climate room at 10 °C. The algae were left to grow for 4 weeks under experimental conditions, and growth media was changed twice a week. After four weeks, the biomass of each glass was weighed and specific growth rates (SGR) were calculated as: $SGR = 100 \times [\ln(W_t/W_0)]/t$, where W_0 and W_t refer to the initial FW of the algae biomass at the beginning of the experiment and the biomass (FW) after t days of experiment, respectively. For calculation of total solids (TS), sub-samples were freeze-dried at - 40 °C and TS calculated as TS: DW-FW*100 %, where FW refers to the fresh weight of a sample, and DW refers to the weight of the freeze-dried sample. For pigment analysis, fresh tissue samples were kept dark at - 20 °C until analysis.

2.2 Pigment determination in *P. palmata*

2.2.1 Extraction method

A simple and rapid extraction procedure was applied in order to minimize the exposure of the samples to light, heat and air. Fresh *P. palmata* samples were ground using mortar and pestle under liquid nitrogen. 5 g of fine powder was weighted and extracted using 20 mL of acetonitrile (VWR Prolabo, Denmark). 0.1 % (w/v) butylated hydroxytoluene (BHT) (Sigma-Aldrich, Germany), was used as antioxidant both in standard solution and during extraction step. Extracted solvent was filtered through 0.45 µm syringe filters before

injecting to High Performance Liquid Chromatography (Agilent 1200 series). All experiments were performed in dim light.

2.2.2 High Performance Liquid Chromatography

Separations were performed on a reverse phase phenomenex C30 column (150 × 4.6 mm i.d., particle size 5 µm) at 30 °C. HPLC was equipped with a photodiode array detector. Methanol and isopropanol/hexane (50:50, v/v) was used as mobile phase at the flow rate of 1 mL min^{-1} (Gentili and Caretti, 2011). Sample injection volume of 20 µL and UV wavelength range of 450 – 560 nm was used for detection. Peak assignment was performed by comparing the retention times and line spectral properties obtained from photodiode array detection with lutein and β-carotene standards (Extrasynthese, France). Peak for Chlorophyll a (chl a) was identified on the basis of UV spectrum and comparing with the available data in the literature. All solvents were of HPLC grade (VWR Prolabo, Denmark). Relative concentration of each pigment during six treatments was calculated based on the relative peak area. For absolute quantification, use of standard of known concentration is required.

2.3 Statistical analysis

Two-way analysis of variance (ANOVA) was used for comparison of growth rates and to test the significance of the differences of the treatments at the 95 % confidence level except when stated otherwise.

3. Results and Discussion

3.1 Growth rates

The specific growth rates (SGR) of *P*. *palmata* in this study ranged between 0.20 ± 0.04 and 0.71 ± 0.05 % fresh weight per day (FW d^{-1}) (Figure 1). A significant interaction between the effects of nutrient concentration and light regime was observed, indicating that the positive effect of the white light on the SGR was greater when the algae had access to high nutrient concentrations. Overall there was a positive effect of nutrient concentration on SGR, and also an effect of light quality with significantly higher SGR observed in the algae growing under WL, as compared to RL and BL. There was no significant difference in SGR between the algae cultivated in RL and BL and also there was no significant effect of nutrient or light on the total solid % (TS %) of the algae (Data not shown).

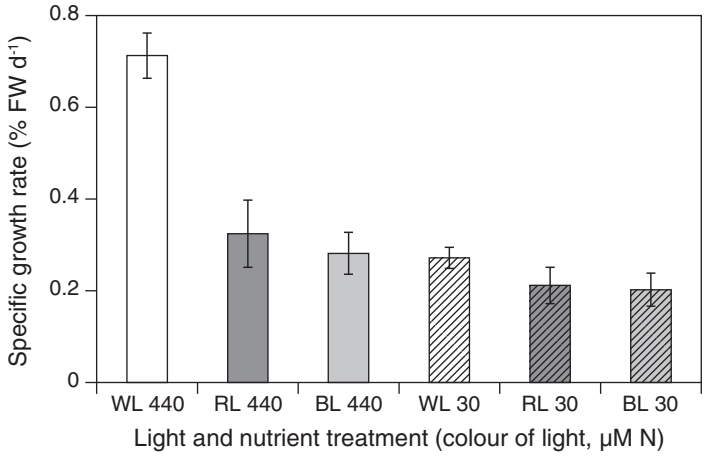

Figure 1: Specific growth rates of P. palmata under different conditions of light (WL: white light, RL: red light, BL: blue light) and nutrients (30 and 440 µM of nitrate, respectively). Data are represented as average ± SE, n = 3

The specific growth rates achieved in this study are relatively low compared to other laboratory studies with light of different spectral composition and even lower light intensities than applied in this study (Sagert and Schubert, 1995b). In the mentioned study, observed growth rates of *P. palmata* were ranging between 8 and 20 % FW d^{-1}. In tank cultivation studies, SGR of 12 % DW d^{-1} have been reported (Pang and Lüning, 2004). Despite the low growth rates, the response in SGR observed between the different light qualities are in agreement with the findings of similar studies, with WL giving the highest SGR, and no significant difference between the SGR in RL or BL (Sagert and Schubert, 1995a). In another red alga, *Porphyra*

umbilicalis, RL was demonstrated to induce significantly higher SGR compared to BL (Figueroa et al, 1995). Our results support results by Sagert and Schubert (1995a) that *Palmaria palmata* can grow with light of a spectral composition preferentially exciting one of the two photosystems, here red and blue light, both being absorbed primarily by Photo System I (PSI). However, for both light qualities at high nitrate composition, SGR is lower than when both photo systems are involved.

3.2 Pigment concentration

The relative concentration of three different pigments in *P. palmata* under six cultivation conditions (three light regimes and two concentrations of nitrogen) was investigated. The influence of nitrogen load had significantly more impact than light on the pigment concentrations of *P. palmata*.

In the algae cultivated at the low nitrate concentrations, the relative concentration of the pigments significantly decreased for all light regimes as compared to the algae cultivated at the high nitrate concentration (Figure 2). This is in agreement with findings from studies on higher plants (Kopsell et al., 2007). The same trend was also found for the microalga *Dunaliella salina,* in which the chlorophyll concentration decreased with decreasing availability of nitrate (Pisal and Lele, 2005). As one chlorophyll molecule contains 4 nitrogen atoms, cell organelles cannot synthesize chlorophyll in the absence of sufficient nitrogen, and therefore an observed decline in chlorophyll content may be interpreted as a consequence of nitrogen starvation. Based on the carotenoid structure on the other hand, nitrogen availability should not directly affect carotenoid concentrations, since carotenoids do not contain nitrogen. Indirectly however, a decrease in nitrogen followed by a decrease in chlorophyll a could cause a lower capacity to absorb the incoming light in photosynthesis. Consequently, more photo-damage could stimulate an increase in carotenoids as antioxidants. It has been demonstrated that nitrogen load and form does have an effect on carotenoid concentrations in higher plants and some similarities in behavior of carotenoids and chlorophyll have been reported for certain plants (Kopsell et al., 2007) and could therefore also be expected for macroalgae.

For the algae cultivated at the low nitrate concentration, the different light treatment had no significant effect on the pigment concentrations except for an increase in β-carotene (confidence level of 90 %) potentially as mentioned a stimulation of antioxidant production (Figure 2b). At high nitrate concentrations, BL induced the highest increase in algae lutein content whereas no significant difference in the increase was observed between the algae cultivated in RL and WL (Figure 2a). Lutein is mainly associated with photosystem II (PSII) and has a primary role in higher plants as a photo-protective pigment. In BL, there may be an enhanced need for photo-protection of PSII, due to increased transfer of energy to PSII from other accessory pigments, the red algae phycobiliproteins, that also absorb light in the blue regions. This may explain the high lutein content in *P. palmata* cultivated in BL.

The concentration of β-carotene was significantly higher in *P. palmata* cultivated in WL, while WL and BL had the relatively similar effect on the relative concentration of chlorophyll a (Figure 2c). Sagert and Schubert (1995a; 1995b) also observed how blue light-adapted *P. palmata* contained almost two-fold higher concentrations of carotenoids and chl a than algae adapted to WL, RL, and yellow light (YL). This agrees well with findings from other red algae: *Porphyridium cruentum* and *Porphyra umbilicalis* (Lopez-Figueroa and Niell, 1990). In contrast to their observation, we found clear enhancement of the β-carotene content in alga cultivated under WL.

In a cultivation perspective our results indicate that for biomass production and for production of a biomass rich in β-carotene and chlorophyll a, WL is more efficient than RL or BL. A post-cultivation adaptation to BL though could be used to increase the content of lutein and chlorophyll a in the biomass. The contents of chlorophyll a, β-carotene and lutein are all larger in algae cultivated with access to sufficient nitrate. In tank cultivation of *P. palmata*, the suggested manipulations of external parameters are possible, whereas in open water cultivation access to nutrients as well as blue light to stimulate lutein production could potentially be achieved by increasing the depth of the cultivation lines.

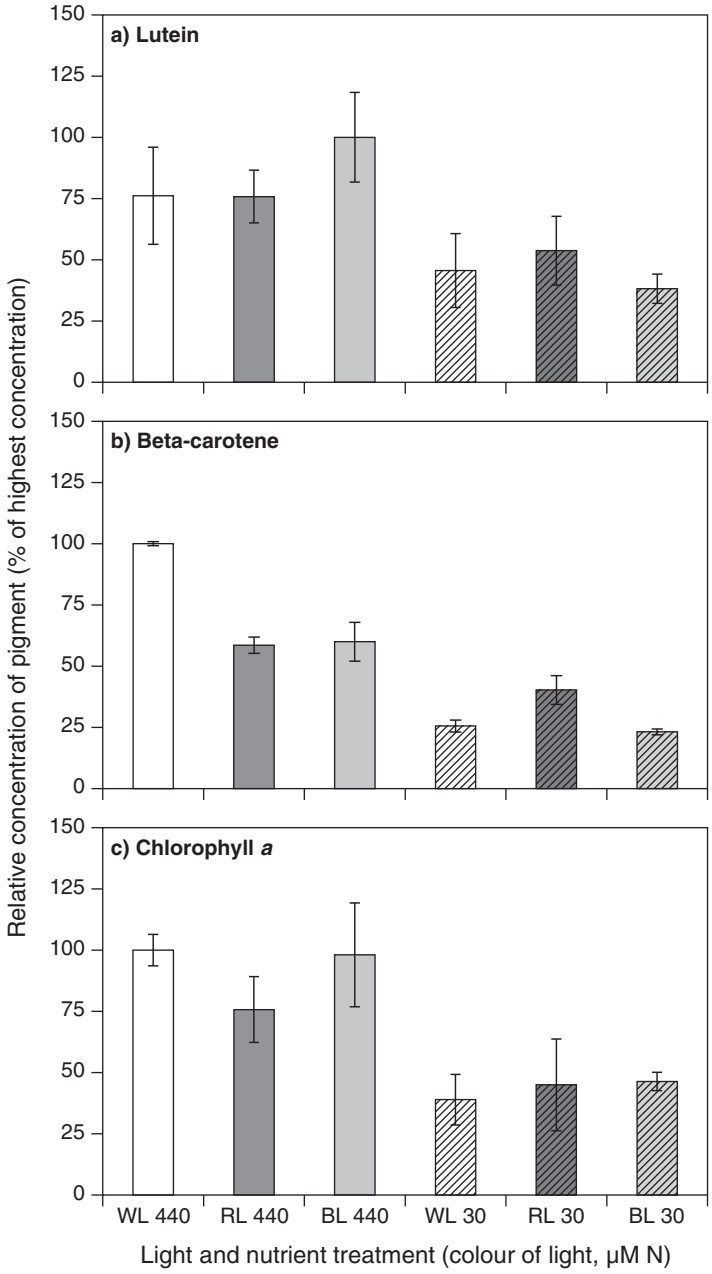

Figure 2: Normalized relative concentration of pigments (a) Lutein, b) Beta-carotene, and c) Chlorophyll a of P. palmata under different conditions of light (WL: white light, RL: red light, BL: blue light) and nutrients (30 and 440 µM of nitrate, respectively). Data are represented as average ± SE, n = 3

4. Conclusion

Palmaria palmata was cultivated in laboratory scale to investigate the influence of nitrate availability and light spectral composition on SGR as well as carotenoid and chlorophyll concentration. Significantly higher SGR was observed when exposing the algae to WL compared to RL and BL at high nitrate concentrations. Increasing the nitrogen load led to significant increase in the relative pigment concentration for carotenoids and chlorophyll a in all light regimes investigated whereas no significant difference was observed at low nitrate concentrations. The highest lutein content was observed in alga grown under BL, while the appropriate light regime for β-carotene production was WL. For chlorophyll no significant difference in concentration could be seen between WL and BL conditions, though RL seems to induce lower chlorophyll a content than WL at high nitrate concentrations. Therefore, depending on consumer demands for different

natural products, light composition and nitrate composition can be reconsidered in marine production programs to increase the productions of specific nutritionally important pigments.

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