



Characterization of the photosynthetic conditions and pigment profiles of the colour strains of *Hypnea musciformis* from field-collected and *in vitro* cultured samples

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Abstract: *Hypnea musciformis* (Wulfen) JV Lamour. is a species of great economic interest as it produces κ -carrageenan and has shown biological activities against HIV and HSV viruses. This species displays different colour strains in its natural habitat, which may have implications for the biotechnological potential of the species. The aim of this study was to characterize the photosynthetic apparatus and pigment profile of three colour strains of *H. musciformis* (green, brown and red) in their natural habitat and in culture. Chlorophyll *a* fluorescence of photosystem II was measured with a pulse-amplitude modulated fluorometer and pigments were quantified by spectrofluorimetry (chlorophyll *a*) and spectrophotometry (phycobiliproteins). In the natural habitat, we detected significant differences between the colour strains for the following photochemical parameters: the green strain had a higher effective quantum yield (Φ PSII) than the red strain and a higher maximum relative electron transport rate (rETRmax) than the brown and red strains. Saturation irradiances were 1000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (green) and 500 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (brown and red). Concerning *in vitro* culture, the green strain presented the lowest Φ PSII, rETRmax, and α rETR, while the brown strain presented the highest values for these same parameters. The chlorophyll *a* content of the cultured green strain was the lowest. The phycoerythrin contents of the three colour strains were unchanged by either natural or *in vitro* conditions: lower in green, intermediate in brown and higher in the red strain, ensuring the chromatic identity of the strains. Our results suggest that the green strain has a better performance when exposed to high irradiance, but a lower efficiency under low irradiance compared to the brown and red strains.

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Introduction

The photosynthetic capacity of an organism can be characterized by chlorophyll *a* fluorescence analysis (Bolh ar-Nordenkampf &  quist, 1993; Baker, 2008). This is a sensitive method, enabling instant and non-invasive measurements of living organisms in both the field and laboratory. It provides information about the ability of an organism to endure environmental stress by indicating, for example, damage to the photosynthetic apparatus. In photosynthesis, light energy is absorbed by a light-harvesting antenna complex and transferred to the reaction center of photosystem II (PSII) and then to photosystem

I (PSI), both located in the thylakoid membrane - constituting the photochemical event. Excess energy not absorbed by the photochemical event is dissipated as heat and fluorescence, the non-photochemical events. Non-photochemical quenching reduces the effective absorption of energy by the photosystem, thus avoiding damage to the photochemical apparatus (Bolh ar-Nordenkampf &  quist, 1993; Baker, 2008). Both photochemical and non-photochemical events of light dissipation, determined by PSII fluorescence analysis in association with determination of the pigment profile, are valuable tools for the characterization of the photosynthetic apparatus of several organisms (H ader et al., 1997; Bautista & Necchi

2007; Yokoya et al., 2007).

PSII fluorescence, oxygen evolution and pigment profile analysis were used to characterize and evaluate the photosynthetic apparatus of two colour strains of cultured *Hypnea musciformis* (Wulfen) JV Lamour. (Yokoya et al., 2007). The strains were obtained from a single thallus with brown and green branches, the latter probably being a mutant (Yokoya et al., 2007). Both strains showed similar potential quantum yield of photosystem II (Fv/Fm) and effective quantum yield ($F/Fm' = \Phi_{PSII}$) (Yokoya et al., 2007). However, the brown strain showed a higher maximum photosynthetic rate and photosynthetic efficiency than the green strain. The pigment profile also differed between the colour strains; for instance, no phycoerythrin was detected in the green strain. Phycoerythrin gives a red colour to algae and can completely mask the green colour of chlorophyll *a*. Phycoerythrin plays an important role in the light-harvesting complexes (Neveux et al., 2006) and is one of the main nitrogen reserves in algae (Martínez & Rico, 2002).

There are natural populations of *H. musciformis* in which the thalli have a single, apparently fixed colour at a given beach, such as the green epilithic colour strains and brown epiphytic colour strains in the State of Rio de Janeiro, Brazil (Reis & Yoneshigue-Valentin, 1998). At another beach, only the red epilithic strain was found. The occurrence of such colour strains is a reflection of the relative concentration of chlorophyll *a* and accessory pigments, sometimes of a deficiency of the latter (Yokoya et al., 2007). The occurrence of colour strains is common among Rhodophyta in genera such as *Gracilaria* Greville (Costa & Plastino, 2011; Ursi & Plastino, 2001); *Euclima* J. Agardh and *Kappaphycus* Doty (Gerung & Ohno, 1997). Colour strains can be a result of either phenotypic acclimation to various light intensities (photoacclimation) or different genotypic adaptations (Kirk, 1994). Specimens with different pigment compositions may present different biochemical and physiological responses to abiotic factors (Kursar et al., 1983; Yokoya et al., 2007), different compositions of polysaccharides (Guimarães, 2000) and also different growth rates and pattern of photosynthesis (Guimarães, 2000; Ursi & Plastino, 2001; Yokoya et al., 2007).

Photosynthetic characterization of the different naturally occurring colour strains of *H. musciformis* is of major importance for environmentally sustainable exploitation of such resources. *H. musciformis* has great economic value for the production of κ -carrageenan, an industrially important sulfated polysaccharide (Oliveira, 1998; Reis et al., 2006), and has shown antiviral activity against HSV (herpes simplex virus) and HIV (human immunodeficiency virus) (Neushul, 1990). *H. musciformis* also contains lectins, which have anti-inflammatory and hemagglutinating activity, besides being useful in cancer diagnosis (Nagano et al., 2002; Nagano et al.,

2005). Extracts of *H. musciformis* also proved to have anthelmintic, anti-inflammatory, myo-relaxing and anti-fungal action (Salimabi, 1980; Davies et al., 1984; Melo et al., 1997).

This study characterizes the photosynthetic apparatus and the pigment profiles of three colour strains of *H. musciformis*-green, brown, and red in both the field and in *in vitro* culture.

Materials and Methods

Algal material

Three colour strains of *Hypnea musciformis* (Wulfen) JV Lamour. were collected at the sublittoral fringe in Rio de Janeiro (RJ), Brazil. Green and brown strains were collected at Praia Rasa, Armação de Búzios (22°44'02"S-41°57'29"W), and the red strain at Prainha, Arraial do Cabo (22°57'30"S-42°01'30"W). Some samples were kept alive for fluorescence analysis in the field and in culture, while others samples were kept in a freezer for pigment analysis. To implement the culture, the apical segment of each colour strain was propagated by fragmentation of a single tetrasporophyte thallus, which yielded the clones. Three unialgal cultures were obtained from different colour strain clones using the protocol of Fernandes et al. (2011). The clones were maintained in controlled culture conditions with an irradiance of $35 \pm 5 \mu E \cdot m^{-2} \cdot s^{-1}$ (measured with a LICOR LI-190 SA quantummeter) at $22 \pm 1 \text{ } ^\circ C$ in 12.5% Von Stosch stagnated culture medium (Edwards, 1970) with a salinity of 32 and a 12 h photoperiod. Ten apices of each colour strain (7 mm long) were placed in different flasks containing 500 mL of culture medium.

Analysis of the photosynthetic apparatus

The photosynthetic apparatus of the three colour strains of *H. musciformis* from the field and the *in vitro* culture was evaluated by measuring PSII fluorescence (FMS-2, Hansatech Instruments Ltd., KingsLynn, UK) with an amplified and modulated amber (540 nm) light pulse. Maximal quantum yield ($Fv/Fm = (Fm - Fo)/Fm$) were obtained after 30 min of dark adaptation (Mouget & Tremblim, 2002). Initial fluorescence was obtained using modulated light $< 0.05 \mu E \cdot m^{-2} \cdot s^{-1}$ (Mouget & Tremblim, 2002), and maximum fluorescence by using a saturating light pulse (0.7 s; $15,300 \mu E \cdot m^{-2} \cdot s^{-1}$). Then, the same samples were measured in the light to evaluate the effective quantum yield ($\Phi_{PSII} = (Fm' - Fs)/Fm'$) and non-photochemical quenching ($NPQ = (Fm - Fm')/Fm'$) (Mouget & Tremblim, 2002). Rapid light curves (RLC) were obtained from the relative electron transport rate ($rETR = \Phi_{PSII} \cdot PAR \cdot 0.5$) (Durako & Kunzelman, 2002; Ralph & Gademann, 2005). Light-adapted measurements

were made on apical segments exposed to actinic light with a photosynthetically active radiation (PAR) range of 18-1033 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

All parameters reported were obtained approximately one hour after the thalli had been collected in the field, while measurements of *in vitro* cultured thalli were made 30 min after the lights were turned on. All measurements were made in a crystallizing dish with specimens immersed in 100 mL of the original medium: seawater for field specimens or Von Stosch culture medium for *in vitro* specimens. The light-emitting extremity of the fibre optic was immersed in the medium at a distance of 5 mm from the thallus apex. Four replicates were measured for each different strain/treatment.

Pigment extraction and profile

Chlorophyll *a* (Chl *a*) and phycobiliproteins (PHB) were analysed in the field samples and *in vitro* cultured samples. Wet frozen thalli were used ($n=4$); 20-80 mg of thalli were used for chlorophyll extraction and 150-270 mg for PHB extraction. Extraction procedures were performed in the dark. For chlorophyll extraction, thalli were macerated wrapped in a GF/F glass microfiber filter (Whatman) in a test tube with 6 mL of acetone 90% (Merck). The tubes were kept at 4 °C for 18 h for total extraction of Chl *a*, centrifuged at 1800 rpm for 5 min and the supernatant analysed by spectrofluorimetry (Varian Cary Eclipse spectrofluorimeter) according to Neveux & Lantoiné (1993), with the following modifications: 1) acquisition of a series of fifteen fluorescence emission spectra ranging in excitation wavelengths from 390-432 nm every 3 nm. From each emission spectrum, we recovered a value every 2 nm between 659 to 715 nm, thus totaling 435 measured fluorescence data points. The fluorescence excitation and emission slit widths were set at 5 and 10 nm, respectively, and the photomultiplier voltage to 800 V; 2) elimination of negative solutions was performed by using the least-squares approximation technique.

For PHB extraction, thalli were ground in a mortar with 6 mL of phosphate buffer (0.1 M, pH 6.5) and poured into a test tube. The PHBs extracted were phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC). Prior to analysis, the extracts were maintained at 4 °C for 16 h and centrifuged at 1800 x *g* for 5 min. The concentrations of PE, PC and APC were obtained by measuring the absorbance of the supernatants with a spectrophotometer (80 Cirrus ST, Femto) and using the equations of Kursar et al. (1983).

Data analysis

Following normality (Kolmogorov-Smirnov's) and homoscedasticity (Levene's) tests, data were analysed

using factorial analysis of variance (one-way and two-way ANOVA) and, if necessary, Tukey's test ($p<0.05\%$) using Statistica software v.7 (StatSoft, Inc., 2004).

Results and Discussion

Photosynthetic parameters

The potential quantum yield (F_v/F_m) of the three colour strains did not differ between field samples ($F=0.45$, $p=0.645$) or *in vitro* cultured specimens ($F=0.57$, $p=0.577$) (Table 1). Previous studies reported similar F_v/F_m values under natural conditions (around 0.6 r.u.) for Rhodophyta species (Mouget & Tremblim, 2002; Figueroa et al., 2009; Chaloub et al., 2010). Higher F_v/F_m values were reported for *in vitro* cultured *Hypnea musciformis* (Wulfen) JV Lamour. strains (Yokoya et al., 2007) than those reported here. In the natural habitat, the green colour strain had the highest effective quantum yield (Φ_{PSII}), while the red strain had the lowest ($F=79.12$, $p<0.010$). Under the low light conditions of the *in vitro* culture, the green colour strain had the lowest Φ_{PSII} among the colour strains ($F=68.02$, $p<0.010$) (Figure 1). The rapid light curves are shown in Figure 1. In the natural habitat, the green strain showed the highest $rETR_{max}$ ($F=10.95$, $p=0.002$) (Figure 2) and the highest light saturation point (around 1,000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). However, the photosynthetic efficiencies (α $rETR$) were similar among the three strains (Table 1). The brown and red strains showed similar light saturation points, approximately 50% lower than that of the green strain (around 500 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). In culture, the brown strain had the highest $rETR_{max}$ and the green strain the lowest ($F=18.40$, $p<0.001$). The same pattern was observed for α $rETR$. All colour strains saturated at around 56 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The green strain was the most efficient under natural high light conditions among the three colour strains and showed the lowest photosynthetic yield under low light culture condition. Non-photochemical quenching (NPQ) drains excitation energy and prevents damage to the photochemical apparatus (Mouget & Tremblim, 2002; Baker, 2008). The NPQ was equivalent for all colour strains under both growth conditions, although, as expected, considerably lower in culture (Figure 3). Under low light, only a small portion of the energy entering the photosynthetic process (3%) is dissipated through heat emission and fluorescence (Bolhár-Nordenkamp & Oquist, 1993). Large amounts of light energy cannot be fully utilized by the photochemical process and the excess energy therefore has to be dissipated (Falkowski & Raven, 2007). The photosynthetic characterization of the three strains suggests that the green strain copes more efficiently under high light conditions but less efficiently under low light conditions than the brown and red strains. Additionally, the Φ_{PSII} and $rETR_{max}$ parameters appear to be more sensitive than F_v/F_m for the evaluation of the

photochemical performance of the colour strains because it was able to differentiate between them both in situ and *in vitro*.

Pigment profiles

The content of Chl *a* did not differ significantly between the three colour strains in the natural habitat ($F=0.67$, $p=0.545$). Under *in vitro* culture conditions, the concentration of Chl *a* differed significantly only between the green strain and the others two colour strains ($F=112.89$, $p<0.001$), with a reduction of 32.6% in the content (Figure 4). The photochemical process is influenced primarily by irradiance and the Chl *a* concentration (Dring, 1982). The observed decrease in

the Chl *a* concentration in the green strain in culture may be related to the lower Φ PSII shown by this strain *in vitro*. Regarding the phycobiliproteins, PE was the major pigment in the three colour strains and, being the dominant pigment in Rhodophyta, (Kirk, 1994; Van den Hoek et al., 1995). The colour of each strain is directly related to the observed PE concentration gradient (Figure 4). However, only the green strain differed from the other two strains (Field: $F=28.55$, $p<0.001$; *In vitro* $F=16.31$, $p=0.004$). The phycoerythrin content was lower in the *in vitro* cultured strains compared to the natural habitat: a 13.81% decrease for the green, 53.53% for the brown and 61.15% for the red strain (Figure 4). Importantly, the green colour strain of *H. musciformis*, unlike other green strains found in Rhodophyta (Yokoya et al., 2007; Costa & Plastino,

Table 1. Maximal quantum yield (Fv/Fm-r.u), relative electron transport rate (rETR_{max}- $\mu\text{E m}^{-2}\text{s}^{-1}$), photosynthetic efficiency (α rETR) of green (Grn), brown (Bro) and red (Red) colour strains of *Hypnea musciformis* in the field (F) and under culture (C) conditions. Values shown are the means and (\pm) standard deviations. The letters indicate significant differences according to two-way ANOVA ($p<0.05$).

Parameters	Field			Culture		
	Grn	Bro	Red	Grn	Bro	Red
Fv/Fm	0.62 \pm 0.03	0.61 \pm 0.03	0.60 \pm 0.04	0.44 \pm 0.08	0.49 \pm 0.06	0.46 \pm 0.06
rETR _{max}	39.78 \pm 1.03	27.05 \pm 4.44	23.57 \pm 7.64	4.53 \pm 0.43a	10.37 \pm 2.43b	6.80 \pm 0.99ab
(α rETR)	0.27 \pm 0.2	0.23 \pm 0.02	0.21 \pm 0.05	0.05 \pm 0.02a	0.19 \pm 0.02b	0.14 \pm 0.02c

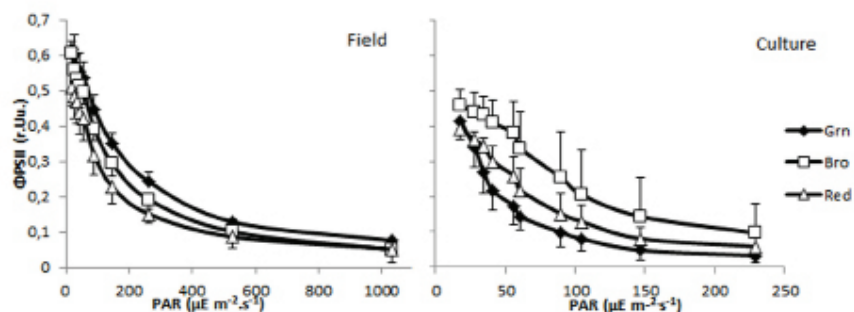


Figure 1. Effective quantum yield (Φ PSII) as a function of the irradiance for green (Grn), brown (Bro) and red (Red) colour strains of *Hypnea musciformis* in the field and under culture conditions. The symbols are the means and the bars indicate the standard deviations.

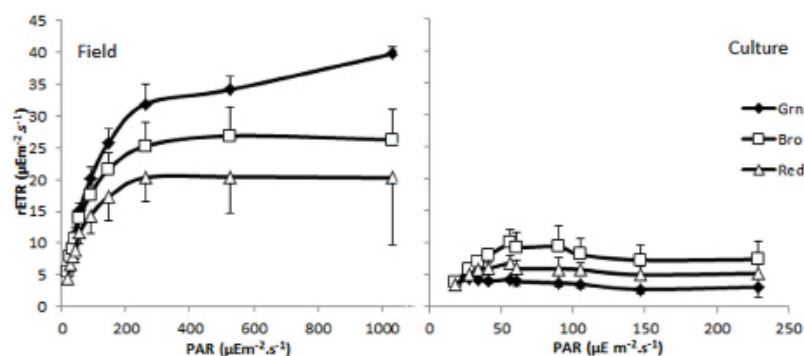


Figure 2. Rapid light curves of the relative electron transport rate (rETR) as a function of the irradiance for green (Grn), brown (Bro) and red (Red) colour strains of *Hypnea musciformis* in the field and under culture conditions. The symbols are the means and the bars indicate the standard deviations.

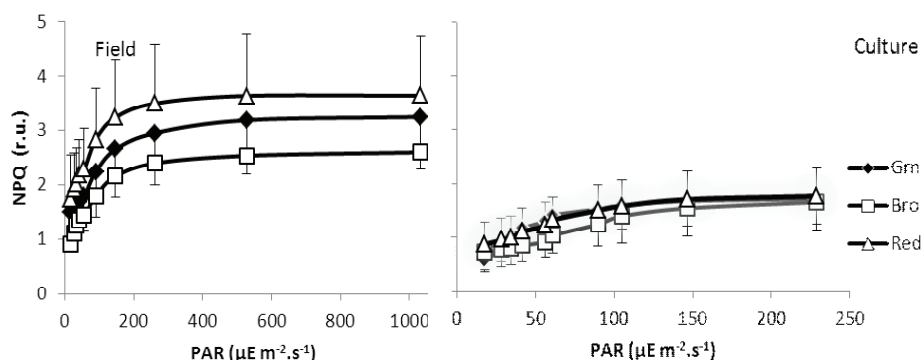


Figure 3. Non-photochemical quenching (NPQ) as a function of the irradiance for green (Grn), brown (Bro) and red (Red) colour strains of *Hypnea musciformis* in the field and under culture conditions. The symbols are the means and the bars indicate the standard deviations.

2011) showed the presence of PE. Regarding the content of PC, there was no evidence of significant differences between the strains for field samples ($F=5.07$, $p=0.051$) or in the *in vitro* culture ($F=2.08$, $p=0.206$). There was a reduction of 76.66% in the concentration of PC for the *in vitro* cultured strains in relation to the field ones (Figure 4). Aphyococyanin concentrations in the green strain differed from that of the red strain in the natural habitat ($F=6.91$, $p=0.278$). Under culture conditions, the green strain had a concentration of APC significantly different from that of the other two strains ($F=112.89$, $p<0.001$). Compared to the natural habitat, the concentration of APC was reduced 48.39% in the three colour strains in culture. The relative contributions of each pigment for the three strains are shown in Figure 5.

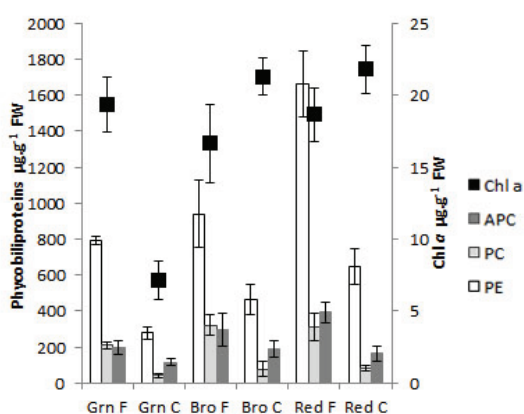


Figure 4. Concentrations ($\mu\text{g.g}^{-1}$ fresh weight) of chlorophyll a and the phycobiliproteins (phycoerythrin (PE), phycocyanin (PC) and aphyococyanin (APC)) for green (Grn), brown (Bro) and red (Red) colour strains of *Hypnea musciformis* in the field (F) and under culture (C) conditions.

During approximately one year of *in vitro* culture, the colour of the three strains was maintained, although less intense in the brown and the red strains

compared to the natural habitat. The maintenance of the proportionality of phycobiliproteins, especially phycoerythrin, ensures the chromatic identity of the colour strains. This was found previously in other Rhodophyta colour strains (Costa & Plastino, 2001, 2011; Yokoya et al., 2007). This suggests that different colours are not the result of chromatic adaptation to naturally occurring environmental variations. To better characterize these strains, morphological and molecular analysis, as well as the photosynthetic responses to variations in the quality and/or quantity of light are needed.

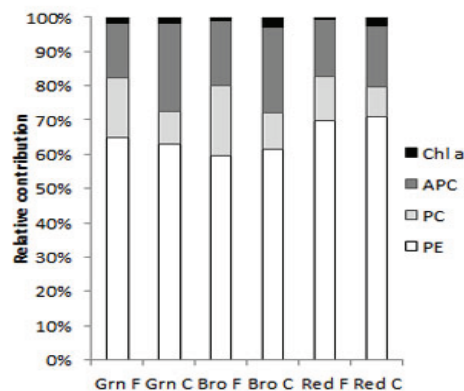


Figure 5. Relative contributions of chlorophyll a and the phycobiliproteins-phycoerythrin (PE), phycocyanin (PC) and aphyococyanin (APC) for green (Grn), brown (Bro) and red (Red) colour strains of *Hypnea musciformis* in the field (F) and under culture (C) conditions.

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