

Effect of Light Regime on the N-ammonium Removal by the Red Algae *Gracilaria vermiculophylla*

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Abstract: The objective of the study was to evaluate the effect of 3 wavelengths (400-700, 455-475 and 620-630 nm) and 3 photoperiods (12:12, 16:08 and 24:00 h) on the ammonium removal and growth of *Gracilaria vermiculophylla*. In a first experiment (8 h), the total ammonium nitrogen removal rate (RR_{TAN}) ranged from 11.63±3.21 to 15.45±3.70 µmol g DW/h, while the efficiency rate of removal, ER_{TAN} varied from 32.40±8.93 to 43.03±10.31%. In a second experiment (8 d), the Specific Growth Rate (SGR) of the macroalgae ranged from 7.24±1.35 to 13.24±1.14% /day. At the end of the trial, the N content in the tissues of *G. vermiculophylla* varied from 1.49±0.14 to 2.53±0.45 g/100 g DW. The highest biomass harvested and N incorporation rate (NIR in mg.N/L.d) corresponded to the photoperiod 16:08 at a wavelength of 620-630 nm. It is concluded that photoperiod and light wavelength had a significant effect on SGR and N content in the tissues of the macroalgae.

Keywords: Bioremediation, *Gracilaria vermiculophylla*, nitrogen removal, photoperiod, wavelength

INTRODUCTION

The first source of Nitrogen (N) inlet to an aquaculture system is the formulated feed. In energetic terms, NH₃ is considered the cheaper form of nitrogen excretion (Forster and Goldstein, 1969) and consequently is the main product of nitrogen metabolism. In crustaceans as in many other organisms the ammonium metabolites, especially NH₃, must not remain into the cells and tissues due to their high toxicity (Frias-Espericueta and Paez-Osuna, 2001) and is highly recommendable to implement strategies for removing them as much as possible from the farming system. The macroalgae, are capable to assimilate diverse forms of N, including NH₃, NO₂ and NO₃; this make them an excellent candidates to be incorporated in Integrated Multitrophic Aquaculture systems (IMTA) (Troell *et al.*, 2003; Neori *et al.*, 2004).

In the culture tanks, NH₃ production is continuous and the macro algae must assimilate it at a rate that avoids its accumulation. Nutrients and light are the main factors affecting the macroalgae metabolism. It is reported that photoperiod and temperature have an influence on the gametogenesis of the Rhodophyta (Guiry and Cunningham, 1984) and that the light intensity and photoperiod affect the growth and nutrient

assimilation of germlings in *Padina boergesenii* (Vasuky *et al.*, 2001). Otherwise, Lobban and Harrison (1997) documented that the blue light is required for a normal development and improve the growth, while the red light favors the cellular division and growth (Figueroa *et al.*, 1995).

In a previous study Abreu *et al.* (2011) found that the temperature and light were the main environmental factors that controlled the growth of *G. vermiculophylla* cultured in an integrated culture and suggested that the farming conditions require adjustments in order to promote a high performance for the macroalgae. To evaluate the feasibility of maximizing the biofiltration capacity of these specie, two experiments were developed. The objective was to evaluate the effect of three photoperiods and three light wavelengths on the total ammonia nitrogen removal (TAN: NH₃+NH₄⁺) rate by *G. vermiculophylla*.

MATERIALS AND METHODS

This study was conducted during 2010, in Sonora State (Northwest of Mexico). New branches of *G. vermiculophylla* were collected at the beginning of the spring in the east coast of the Gulf of California, (26°29'22" N and 109°15'55" W) and transferred to the laboratory of Center for Advanced Studies of Sonora State (CESUES) to be acclimated. They were washed

with marine sterilized water and maintained outdoor (natural photoperiod) during two days at a density of 0.65 g/L (wet weight: WW) and a salinity of 35 ppt. After this period, the macroalgae were transferred indoor at the same density but a light intensity (irradiance) around 50 $\mu\text{mol foton/m}^2/\text{s}$ (obtained by fluorescent white lamps) and a photoperiod 12:12 (light: darkness). For the nutrition, the medium Von Stosch (Andersen *et al.*, 2005) enriched with ammonium chloride were used up to a concentration of 56 $\mu\text{mol/L}$ of TAN, as recommended by Skriptsova and Miroshnikova (2011). During the macroalgae acclimation, the water temperature was maintained at $25 \pm 0.34^\circ\text{C}$ and the salinity at 35 ppt. Aeration by diffuser stones was applied to maintain turbulence on the water column. After three days under those conditions a first experiment was initiated to evaluate the effect of wavelength on the TAN removal. Later, a second trial was done to evaluate the effect of photoperiod and wavelength on the growth and N incorporation of *G. vermiculophylla*. To start the experiments, apical branches (4-5 cm) of the acclimated macroalgae were taken.

The irradiance was measured by a quantum spherical sensor Li-Cor 193SA; the temperature with a multi-parameter YSI 550A, the pH with a pH meter Denver Instruments UP-10 and the salinity with a refractometer Aquatic Ecosystems®.

Experiment 1: Evaluation of light wavelength on the TAN removal: Three wavelengths were evaluated by triplicate: $\lambda = 400$ to 700 nm (white), $\lambda = 455$ to 475 nm (blue) and $\lambda = 620$ to 630 nm (red). A light intensity around $273 \pm 12 \mu\text{mol photon per m}^2.\text{s}$ (Phooprong *et al.*, 2008) produced by Diodes Emission Lamps (LED) electronically controlled, was applied to the experimental units.

The experimental units consisted of transparent plastic containers with one L of seawater. The stocking density of the macro algae was $12.00 \pm 0.16 \text{ g/L}$ (WW). The water temperature for the culture was maintained at $28 \pm 1^\circ\text{C}$ by means of titanium heaters, (Aquatic Ecosystems®), salinity at 35 ppt and constant aeration.

At the beginning of the experiment, ammonium chloride was added to each experimental unit up to a TAN concentration of 56 $\mu\text{mol/L}$. A sample of 10 mL of water from each experimental unit was taken at 1, 2, 4 and 8 h, as suggested by Kraemer *et al.* (2004). The water samples were stored in an ultra-freezer at -50°C , up to the subsequent analysis. The TAN was measured by the salicylate method (HACH, 2005). To estimate the Dried Weight (DW) the macroalgae was dried in a convection stove (48 h at 60°C).

The TAN removal rate (RR_{TAN}) and the efficiency rate (RE_{TAN}) were calculated by triplicate for the first hour of the trial, according to Mao *et al.* (2009) as:

$$RR_{\text{TAN}} (\mu\text{mol.g/DW.h}) = (C_0 - C_t) V / (W_s t)$$

where,

C_0 : The TAN concentration ($\mu\text{mol/L}$) at the beginning
 C_t : The TAN concentration ($\mu\text{mol/L}$) at the at the t time
 V : The water volume (L)
 W_s : The dry weight of macroalgae (g)
 t : The time t (h)

$$RE_{\text{TAN}} (\%) = (C_0 - C_t) 100 / C_0$$

Experiment 2: Effect of photoperiod and wavelength on the growth and N incorporation of *G. vermiculophylla*: A second trial was conducted for 8 days, using the same 3 wavelengths of the previous experiment, but combined with 3 photoperiods (12:12, 16:08 and 24:00), in a factorial design 3x3, with three replicates by treatment.

At the beginning of the experiment, 4 g (WW) of the macroalgae were placed in transparent glass recipients with 1.0 L of marine water (35 ppt of salinity). The culture medium was the Von Stochs, enriched with NH_4Cl to reach a concentration of 56 $\mu\text{mol/L}$ of TAN. That concentration was reestablished every day. At day 4, a 100% water exchange was done to avoid the proliferation of bacteria and other undesirable organisms. The irradiance was maintained at $230 \pm 9 \mu\text{mol photon m}^2/\text{s}$, the temperature at 25 - 26°C , the salinity at 35 ppt and the pH at 7-7.5. The WW of the macroalgae was measured at day 8 in a digital balance. The specific growth rate was calculated according to Mao *et al.* (2009) as:

$$\text{SGR} (\%/d) = \text{Ln} (W_t / W_0) 100 / t$$

where,

W_0 : The initial WW (g)
 W_t : The WW at time t (g)
 t : The time (d)

At the end of the trial, samples of macroalgae tissue were taken and lyophilized to measure the N incorporated by the Kjeldahl method, using a programmable spectrophotometer DR/5000 to read the transmittance. The N Incorporation Rate (NIR) was calculated based on Mata *et al.* (2010) as:

$$\text{NIR} (\text{mg.N/L.d}) = [(W_t - W_0) N] / (t V)$$

where,

W_0 : The initial DW (g)
 W_t : The final DW (g)
 N : The content of nitrogen in the tissue (DW)
 t : The time (d)
 V : The volume of water (L)

Test of normality and variance homogeneity were done to the data. After to verify data were normal and homoscedastic, in experiment 1, a parametric one way ANOVA (Zar, 1999) was performed to detect differences on the RR_{TAN} and RE_{TAN} among treatments (wavelengths). When differences were found, a Tukey test was applied to compare and rank means. In experiment 2, a two ways ANOVA was applied to compare SGR and NIR of the macroalgae in the treatments (combination of photoperiods and wavelengths). A Tukey test was performed when differences were found. The Statistica 5.1 for Windows (Statsoft, Inc. ®) was used for the analysis.

RESULTS AND DISCUSSION

The TAN concentration in the first trial decreased during the first hour as follow: from 56 $\mu\text{mol/L}$ to 33.57 \pm 3.58 $\mu\text{mol/L}$ at the wavelength of 400-700 nm (white light), to 37.86 \pm 5.00 $\mu\text{mol/L}$ at 455-475 nm (blue light) nm; and to 31.90 \pm 5.77 $\mu\text{mol/L}$ at 620-630 nm (red light). After 8 h the final TAN concentration were: 7.17 \pm 3.08 $\mu\text{mol/L}$ at 620-630 nm; 13.81 \pm 4.31 $\mu\text{mol/L}$ at 400-700 nm and 18.57 \pm .51 $\mu\text{mol/L}$ at 455-475 nm without significant differences among wavelengths.

The TAN concentration at the end of this experiment indicated that the macroalgae tended to take less N as its concentration decreased in the water column (Fig. 1) this phenomenon is consistent with Hernández *et al.* (2002) who documented that the TAN removal kinetic by *Gracilaria gracilis* fit well to the Michaelis-Menten model in which the removal decrease as the nutrient does it in the water column. They reported that after 7 h the initial concentration of 130 $\mu\text{mol}/\text{NH}_4^+$ g DW was totally consumed and that the higher RR_{TAN} occurred during the first 3 h. It has been shown that the kinetics of absorption of N and its transport to the cells has an accelerated phase, afterward the speed of absorption diminishes and eventually stops when the cellular reserves are full (Lartigue and Sherman, 2005).

The TAN removal rate (RR_{TAN}) for the 1st h of the trial ranged from 11.63 \pm 3.21 to 15.45 \pm 3.70; $\mu\text{mol}/\text{g}$ DW/h and no significant differences were observed among treatments (Table 1). The TAN removal efficiency (RE_{TAN}) varied during the 1st h from 32.40 \pm 8.93 to 43.03 \pm 10.31%; while after 8 h ranged from 75.34 \pm 7.69 to 87.19 \pm 5.51%. Our results indicate that in short periods of time the wavelength seems not to affect the removal of TAN. Otherwise, the RR_{TAN} obtained at 25°C were similar to the 13 $\mu\text{mol}/\text{NH}_4^+$ g DW/h reported by Skriptsova and Miroshnikova (2011) at 15°C for the same species.

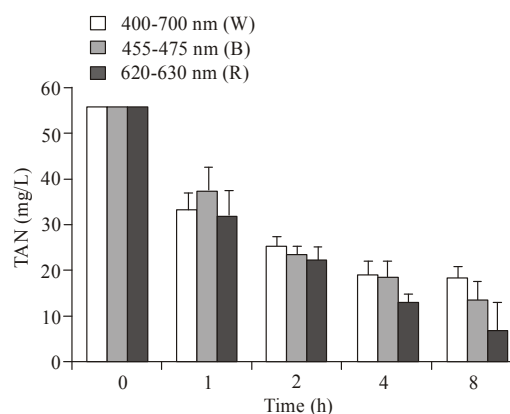


Fig. 1: Means \pm SD of TAN in the treatments W: White light; B: Blue light; R: Red light during the first experiment

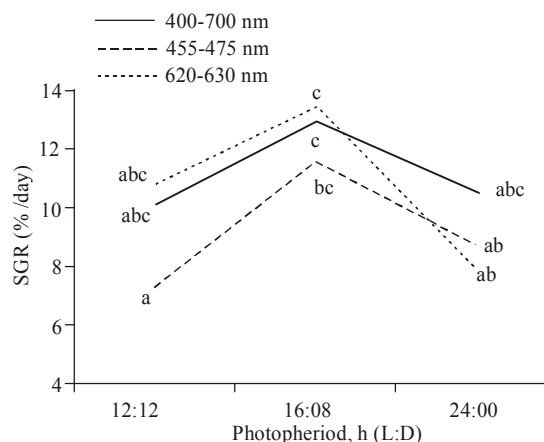


Fig. 2: Specific growth rate (SGR % per day) of *Gracilaria vermiculopylla* farmed at 3 photoperiods and 3 wavelengths Different letters indicate significant differences (p<0.05; a<b<c)

At the end of the second experiment the biomass harvested ranged from 7.17 to 11.57 g/L. The highest value corresponded to the photoperiod 16:08 at a wavelength of 620-630 nm; while the lowest was obtained with the photoperiod of 12:12 and a wavelength of 455-475 (Table 2). The specific growth rate (SGR % per day) in the treatments (combination of photoperiods and wavelengths) varied from 7.24 \pm 1.35 to 13.24 \pm 1.14. The lowest value was found with the photoperiod 12:12 at 455-475 nm. Independently of the wavelength, the greatest biomasses and SGR were obtained with the photoperiod 16:08 (Fig. 2).

The N content on the macroalgae tissues varied from 1.49 \pm 0.14 to 2.53 \pm 0.45 g 100 g/DW. The treatments with photoperiods 12:12 and 24:00 showed to have higher N contents (except at 455-475 nm). The

Table 1: Means±S.D. of the TAN removal rate (RR_{TAN}) and removal efficiency (RE_{TAN}) by *Gracilaria vermiculophylla* at 3 wavelengths

Wavelength (nm)	RR _{TAN} (μmol g/DW h) 0-1 h	RE _{TAN} (%) 0-1 h	RE _{TAN} (%) 0-8 h
400-700	14.38±2.29 ^a	40.05±6.38 ^a	78.74±4.48 ^a
455-475	11.63±3.21 ^a	32.40±8.93 ^a	75.34±7.69 ^a
620-630	15.45±3.70 ^a	43.03±10.31 ^a	87.19±5.51 ^a

Different letters in each column indicate significant differences (p<0.05)

Table 2: Means and S.D. of final wet weight of *Gracilaria vermiculophylla* at 3 photoperiods and 3 wavelengths

Photoperiod light: Darkness (h)	Wavelength		
	White (400-700 nm)	Blue (455-475 nm)	Red (620-630 nm)
12:12	8.97 ^{ab} (0.85)	7.17 ^a (0.75)	9.40 ^{abc} (0.87)
16:08	11.13 ^{cd} (0.57)	10.00 ^{bcd} (0.36)	11.57 ^d (1.06)
24:00	9.23 ^{abc} (1.10)	8.03 ^{ab} (0.95)	7.87 ^{ab} (0.32)

Different letters indicate significant differences (p<0.05; a<b<c<d)

Table 3: Mean±S.D. of N content (g/100g/DW) in *Gracilaria vermiculophylla* tissue at 3 photoperiods and 3 wavelengths

Photoperiod light: Darkness (h)	Wavelength		
	White (400-700 nm)	Blue (455-475 nm)	Red (620-630 nm)
12:12	2.47 ^b ±0.21	2.53 ^b ±0.45	1.90 ^{ab} ±0.10
16:08	1.61 ^a ±0.26	1.76 ^a ±0.12	1.60 ^a ±0.14
24:00	1.49 ^a ±0.14	2.21 ^b ±0.26	2.05 ^{ab} ±0.26

Different letters indicate significant differences (p<0.05; a<b)

Table 4: Means and S.D. of N incorporation rate (mg N/L d) by *Gracilaria vermiculophylla* at 3 photoperiods and 3 wavelengths

Photoperiod light: Darkness (h)	Wavelength		
	White (400-700 nm)	Blue (455-475 nm)	Red (620-630 nm)
12:12	1.91 ^{bc} (0.19)	1.27 ^a (0.17)	1.66 ^{abc} (0.19)
16:08	1.88 ^{bc} (0.20)	1.72 ^{abc} (0.21)	1.96 ^c (0.27)
24:00	1.41 ^{ab} (0.19)	1.69 ^{abc} (0.18)	1.28 ^a (0.09)

Different letters indicate significant differences (p<0.05; a<b<c)

greatest content was observed with the photoperiod 12:12 at a wavelength of 455-475 nm and the lowest with the photoperiod 24:00 at a wavelength of 400-700 nm (Table 3).

For *Porphyra umbilicalis*, Figueroa *et al.* (1995) reported that blue light increased the content of soluble protein as well as the photo protector pigments phycoeritrine and phycocyanine in the tissues, while with red light, the content of those pigments decreased, but the content of carbohydrates and the photosynthetic rate increased. In the present study the maximum growth rate and the lower N content in the macroalgae tissues were found with red light, which is consistent with the results above mentioned. The macroalgae showed better growth rate at a photoperiod of 16:08. The above information suggests that *G. vermiculophylla* respond to a cyclic pattern of light-darkness, which supports the theory that there are molecular watches that control the daily rhythms of the macroalgae (Lüning, 2005).

The N Incorporation Rate (NIR in mg N/L d) showed mean values from 1.27±0.17 to 1.96±0.27. The lowest rate was found with the photoperiod 12:12 at a wavelength of 455-475 nm; while the highest was

observed with photoperiod 16:08, at a wavelength of 620-630 (Table 4).

The differences in the response of macroalgae to wavelength are due to adjustments into the cells to photo acclimation. They modify the synthesis pathways of biomolecules choosing between carbohydrates and lipids or proteins to form the Complexes for Light Harvest (LHC) (Lobban and Harrison, 1997). The acclimation of macroalgae to photoperiod has two components: one is the adjustment of the photosynthetic apparatus, which includes changes in the reaction center as well as number of the LHC and changes in the content of pigments which protect the organisms from the light. The other component includes changes in the morphology of the plant, for instance the formation of ramifications (Marquardt *et al.*, 2010).

In 75% of the interactions wavelength-photoperiod of this experiment, the N incorporation rate was similar, which can be explained because this variable does not depend only on the growth but mostly on the incorporation of protein on the tissues; and both parameters not always are directly correlated.

Based on the results of this study it can be concluded that both, photoperiod and light wavelength

had a significant effect on SGR and N content in tissues of *G. vermiculophylla*. Additionally, it was found that this specie can remove TAN permanently (24 h) if is maintained under constant illumination. These results can be useful to optimize the removal of TAN in the bioremediation systems. As mentioned by Neori *et al.* (2004), for IMTA it is important the growth of the macroalgae, but also their beneficial effect on the water quality of the system by removing nitrogenous metabolites.

ACKNOWLEDGMENT

The study was supported by Program for the improvement of the Teaching Staff (PROMEP; CESUES-EXB-001) project: Biorremediación de efluentes acuícolas mediante el uso de especies endémicas de moluscos filtradores, tapetes de diatomeas bentónicas y vegetales.

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