Photosynthetic responses of *Halimeda scabra* (Chlorophyta, Bryopsidales) to interactive effects of temperature, pH, and nutrients and its carbon pathways (#55374)

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Photosynthetic responses of *Halimeda scabra* (Chlorophyta, Bryopsidales) to interactive effects of temperature, pH, and nutrients and its carbon pathways

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In this study, we evaluated the interactive effects of temperature, pH, and nutrients on the photosynthetic performance in the calcareous tropical macroalga *Halimeda scabra*. A significant interaction between these factors on P_{gross} was found. The highest values of P_{gross} were reached at the highest temperature, pH, and nutrient enrichment tested. The Q_{10} P_{gross}values confirmed the effect of temperature only under nutrient enrichment scenarios. Besides the above, bicarbonate (HCO_3^-) absorption was assessed by the content of carbon stable isotope ($\delta^{13}C$) in algae tissue and by its incorporation into photosynthetic products, as well as by carbonic anhydrase (CA) inhibitors (Acetazolamide, AZ and Ethoxyzolamide, EZ) assays. The results of $\delta^{13}C$ revealed this species uses both, CO_2 and HCO_3^- forms of C_i relying on a CCM. These results were validated by the EZ-AZ inhibition assays in which photosynthesis inhibition was observed, indicating the action of internal CA, whereas AZ inhibitor did not affect P_{max}. The incorporation of ^{13}C isotope into aspartate in light and dark treatments also confirmed photosynthetic and non-photosynthetic the HCO_3^- uptake.

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ABSTRACT

In this study, we evaluated the interactive effects of temperature, pH, and nutrients on ~~the~~ photosynthetic performance in the calcareous tropical macroalga *Halimeda scabra*. A significant interaction ~~between~~ these factors on P_{gross} was found. The highest values of P_{gross} were reached at the highest temperature, pH, and nutrient enrichment tested. The Q_{10} P_{gross} values confirmed the effect of temperature only under nutrient enrichment scenarios. Besides the above, bicarbonate (HCO_3^-) absorption was assessed by the content of carbon stable isotope ($\delta^{13}C$) in algae tissue and by its incorporation into photosynthetic products, as well as by carbonic anhydrase (CA) inhibitors (Acetazolamide, AZ and Ethoxyzolamide, EZ) assays. The results of $\delta^{13}C$ revealed this species uses both, CO_2 and HCO_3^- forms of C_i relying on a CCM. These results were validated by the EZ-AZ inhibition assays in which photosynthesis inhibition was observed, indicating the action of internal CA, whereas AZ inhibitor did not affect P_{max}. The incorporation of ^{13}C isotope into aspartate in light and dark treatments also confirmed photosynthetic and non-photosynthetic the HCO_3^- uptake.

Key index words: Carbonic anhydrase, CCM, ^{13}C isotope, $\delta^{13}C$, *Halimeda scabra*, interactive effects, nutrients, pH, photosynthesis, Q_{10}, temperature.

INTRODUCTION

~~It is known that~~ photosynthetic parameters respond faster to environmental changes than algae C and N content, hence ~~its~~ usefulness in short-term studies (Figueroa et al., 2009). Photochemical and biochemical reactions of photosynthesis continually respond to environmental conditions. Irradiance, temperature, and nutrient concentration including CO_2

levels are among the main environmental factors limiting photosynthesis (Raven & Hurd, 2012; Zweng, Koch & Bowes, 2018). Algal ecophysiology studies have traditionally quantified temperature dependence using the metabolic quotient Q_{10}, which describes the metabolic increase accompanied by an increase of 10°C in an optimal temperature range (Bruno, Carr & O'Connor, 2015; Vásquez-Elizondo & Enríquez, 2016). This quotient Q_{10} has also been used as a proxy to analyze the effect of temperature on nutrient absorption where it was found that by doubling the temperature the rate of nutrient absorption is doubled (Harrison & Hurd, 2001).

For aquatic plants, another limiting factor for photosynthesis is CO_2, since it is the only source of carbon that can be assimilated by the Ribulose 1,5 bisphosphate carboxylase oxygenase enzyme (RuBisCO) (Falkowski & Raven, 2007). At seawater pH (8.1 – 8.3) CO_2 is only between 0.5 – 1% of all dissolved inorganic carbon, while more than 91% is in the form of HCO_3^- and the remaining 8% is in the form of CO_3^{2-} (Hurd et al., 2009; Diaz-Pulido et al., 2016). Moreover, since the diffusion of CO_2 through the cell membrane is slower in water than in air; many algae and higher plants have acquired mechanisms that promote intracellular CO_2 accumulation, allowing photosynthetic organisms to reduce carbon limitation by increasing the concentration of CO_2 in the vicinity of RuBisCO (CO_2 Concentration Mechanisms, CCM). Parallel to this, CCM´s contribute to decreasing photorespiration due to the oxygenase activity of RuBisCO (Ogren, 1984; Enríquez & Rodríguez-Román, 2006; Cornwall, Revill & Hurd, 2015). In general, most algae can acquire inorganic carbon (C_i) for RuBisCO through diffusion and active absorption of both, CO_2 and HCO_3^- (Badger & Price, 1994; Giordano, Beardall & Raven, 2005; Hurd et al., 2009). In many cases, the activity of CCMs has been associated with the direct or indirect use of HCO_3^- (Reiskind, Seamon & Bowes, 1988; Invers et al., 2001; Enríquez & Rodríguez-Román, 2006). Some macroalgae convert bicarbonate (HCO_3^-) into CO_2 extracellularly with carbonic anhydrase (CA) thus CO_2 enters the cell by active transport or diffusion. Other algae incorporate HCO_3^- actively through the cell membrane and, intracellularly, an internal CA converts HCO_3^- into CO_2 (Badger & Price, 1994). The activity of carbonic anhydrases has been widely documented in algae (Reiskind, Seamon & Bowes, 1988; Invers, Perez & Romero, 1999; Enríquez & Rodríguez-Román, 2006) and ~~plays~~ a significant role in CCM´s.

Many studies have ~~been performed to explain~~ the combined effects of environmental variables on algae photosynthetic responses: CO_2 and temperature (Campbell et al., 2016; Kram et al., 2016; Vásquez-Elizondo & Enríquez, 2016); CO_2 and light (Vogel et al., 2015); light and nutrients (Zubia, Freile-Pelegrín & Robledo, 2014); CO_2 and nutrients (Hofmann et al., 2014; Hofmann et al., 2015; Bender-Champ, Diaz-Pulido & Dove, 2017); CO_2, nutrients, and temperature (Stengel et al., 2014), and CO_2, nutrients and light (Celis-Plá et al., 2015). Multiple stressors could have an interactive influence causing complex responses at the physiological and ecological level (Hofmann et al., 2014), which makes them difficult to interpret. Therefore, studies that combine ocean acidification scenarios with other factors such as temperature, light, and nutrient availability are particularly necessary since changes in these parameters are co-

occurring with changes in carbonate chemistry in the seawater (Harley et al., 2012; Hofmann et al., 2014).

Halimeda is a calcifying genus of siphonous green algae (Bryopsidales, Chlorophyta) which are important components of tropical and subtropical reefs and lagoons. Some species of this genus often appear dominating Caribbean coral reefs (Beach et al., 2003; Hofmann et al., 2014) where they contribute as primary producers, food source and habitat, sand production, and coral-reef formation. *Halimeda scabra* Howe is particularly abundant in the front reef and shallow rocky areas of the Caribbean Reefs (Alcolado et al., 2003). Despite the ecological studies above-mentioned, to our knowledge, no previous physiological studies have been reported for this species.

Photosynthetic responses to the combined effect of environmental variables have been studied in some *Halimeda* species, for example in *H. opuntia*, the effect of nutrients and pH (Hofmann et al., 2014; Hofmann et al., 2015), in *H. incrassata* and *H. simulans* the effect of pH and temperature (Campbell et al., 2016), and in *H. opuntia* the effect of pH and light (Vogel et al., 2015). These studies have suggested that an increase in both, CO_2 (low pH) and temperature could have a positive synergistic effect on photosynthetic rates (Kram et al., 2016). However, *Halimeda* responses to high CO_2 have been diverse; ~~while~~ in some species a decrease in photosynthesis ~~rates~~ with the reduction of pH has been observed (Price et al., 2011; Sinutok et al., 2012; Meyer et al., 2016) others have shown the opposite effect (Peach, Koch & Blackwelder, 2016) or a lack of a significant response (Price et al., 2011; Campbell et al,. 2016). In general, there are still insufficient studies on the physiology of the genus *Halimeda* that allow us to understand the diversity of physiological responses to the interactive effects of environmental variables and the mechanisms involved in those responses.

In this study, we ~~hypothesize~~ that a synergistic increase in environmental factors (temperature, pH, and nutrients) ~~enhances~~ *H. scabra* photosynthesis, which absorbs bicarbonate supported by a CCM. We ~~evaluate~~ the interactive effect of temperature, pH, and nutrient ratios on photosynthetic responses of *H. scabra*. Additionally, we determined the C*i* uptake mechanisms by measuring the effect of CA inhibitors on P_{max}, analyzing $\delta^{13}C$ values, and evaluating the incorporation of stable isotope ^{13}C into resulting products of photosynthesis.

MATERIALS AND METHODS

Biological material and culture conditions

H. scabra was collected in February 2017 in Xcalacoco, Quintana Roo, Mexico (20.660035 N, -87.034655 W), where it grows over rocky substrates between 1.5 and 2.0 m depth. Its taxonomic determination was done according to Hillis-Colinvaux (1980). The algae were transported to the laboratory inside Ziploc bags under reduced temperature. At the laboratory, samples were cleaned with seawater to remove epiphytes and placed in 12 L aquarium with filtered seawater (36 PSU, pH 8.2) and kept under constant aeration at 24ºC ~~of temperature.~~ Irradiance was set at 115 µmol photons m^{-2} s^{-1} provided by fluorescent lamps under a 12:12 hours light-dark photoperiod.

Photosynthetic measurements

Photosynthetic responses were evaluated by the light-dark bottles method following the oxygen evolution *versus* irradiance (P-E curves) according to Thomas (1988) using a YSI 5000 Dissolved Oxygen Meter with YSI 5905 BOD Probe (YSI Incorporated Yellow Springs, Ohio, USA). To minimize wound effects, thalli were cut off and weighed 24 hours before oxygen determinations. Apical fragments (0.1 g) were placed in 60 ml Biological Oxygen Demand (BOD) bottles (n = 8), one bottle was used as a blank filled only with filtered seawater. The algae were exposed during one hour to each of seven successive irradiances selected (0, 100, 170, 200, 272, 436, 770 µmol photons m^{-2} s^{-1}) generated by a 500 W halogen lamp and using different mesh size filters until darkness. The temperature was maintained constant placing the BOD bottles in a water bath connected to a water recirculation system (Cole-Parmer® Polystat® Refrigerated Recirculator, USA). Irradiances were measured with a spherical underwater quantum sensor (LI-193SA) connected to LI-1500 Light Sensor Logger (LI-COR, Nebraska, USA). The maximum photosynthesis rate (P_{max}) was calculated as the average of the three highest oxygen production values at saturation irradiances. The dark respiration rate (R_d) was determined as oxygen consumption in total darkness, while the gross photosynthesis (P_{gross}) was determined as net photosynthesis plus light and dark respiration) (Alberte, Hesketh & Baker, 1975). At the end of each assessment the dry weight (DW) was determined, whereby the results were expressed as mg of oxygen g dry weight h^{-1} in 300 ml. All determinations were performed using Instant Ocean® synthetic seawater (Marineland, USA), free of nitrate and phosphate except for nutrient ratio experimental treatments.

To test the effect of temperature, pH, and nutrient concentration ratios on *H. scabra* photosynthesis, a three-factorial design with 36 combinations was used (Zar, 1996) (Table S1). The following factors and levels were tested: 1) temperature at three levels (24, 28 and 33ºC); 2) pH at three levels (7.5, 8.2, and 8.6) obtained by addition HCl/NaOH, and 3) nutrient ratios (KNO_3:K_3PO_4) evaluated at four levels: low (1:0.1 µM), medium (5:0.5 µM), high (10:1.0 µM) and 4) a non-enriched level used as a control treatment. Nutrient ratios and pH values were selected according to prevailing conditions measured at the collecting site, whereas seawater temperatures correspond to mean values found during contrasting seasons (~24°C February – ~30°C August) (Robledo & Freile-Pelegrín, 2005; Rodríguez-Martínez et al., 2010).

Effect of temperature on P_{gross}: photosynthetic Q_{10} coefficient

To better understand photosynthetic responses to temperature we calculated the photosynthetic quotient Q_{10} of P_{gross} under different pH and nutrient conditions. The photosynthetic quotient was determined as the change in the photosynthetic rate within a rise in temperature of 5°C, from 28°C (T1) to 33°C (T2) according to the following formula:

$$Q_{10} = (\text{Rate 2}/\text{Rate 1})^{(10/\text{T2-T1})}$$

where, Rate 1 and Rate 2 were reaction rates measured at temperatures T1 and T2, respectively (Wernberg et al., 2016).

Inorganic carbon pathways

Bicarbonate (HCO_3^-) uptake for photosynthesis were assessed through three techniques: (1) carbon stable isotope ($\delta^{13}C$) values in algal tissue (2) CA inhibitor effects on P_{max}, and (3) ^{13}C stable isotope uptake and its incorporation into resulting products of photosynthesis.

Carbon stable isotope ($\delta^{13}C$) values in tissue from field samples

Whole thalli were carefully washed and decalcified in hydrochloric acid (HCl) at 0.6 M for 8 hours, with hourly changes until full bubbling cessation. Afterward, the material was rinsed with distilled water and dried for 24 hours at 70°C. The dried material was ground in a mortar and sieved. Samples of five mg were weighed on analytical balance (precision of 0.0001 g) and individually packaged in microcapsules (5 x 9 mm) for mass spectrophotometer isotopic analysis in the Stable Isotropy laboratory at the University of California at Davis, CA, USA.

Carbonic anhydrase inhibition assays

Two CA inhibitors were used in this study: a) dextran-bound acetazolamide (AZ) that does not penetrate into the cell and only inhibits extracellular CA (Bjork et al., 1992), and b) 6-ethoxyzolamide (EZ) that penetrates through the cell wall and membranes, and inhibits both external and internal CA (Bjork et al., 1992). AZ and EZ were dissolved in 0.05 N NaOH to a final concentration of 0.1 g ml^{-1} and 10 mM respectively (Bjork et al., 1992). Experimental treatments were prepared with filtered and sterilized seawater from the collecting area. The inhibitors were added to the experimental seawater before the incubations to obtain a final inhibitor concentration of 100 µM (Bjork et al., 1992). Photosynthesis rates were tested under four ~~treatment:~~ 1) addition of AZ; 2) the addition of EZ; 3) the combination of both, AZ and EZ and 4) a control treatment with seawater without inhibitors. Maximum photosynthesis (P-E curves) was measured as previously described but at 28°C of temperature.

^{13}C Labeling for the incorporation of $NaH^{13}CO_3$ into photosynthetic products

Initially, inorganic carbon was removed from filtered and sterilized seawater by reducing pH to ~ 4 adding HCl 0.5 M and nitrogen bubbling for 5 hours, subsequently the pH was raised to 8.2 adding NaOH 0.5 M (Invers et al., 2001; Zou, 2014). Afterward, 1.6 g L^{-1} of $NaH^{13}CO_3$ (isotope ^{13}C 99% Aldrich) was added. *H. scabra* thalli fragments (2 g) were placed in hermetically sealed 250 mL BOD bottles containing seawater previously prepared with ^{13}C isotope and maintained for 24 hours at 28°C of temperature under light saturation (278 µmol photons m^{-2} s^{-1}). Three photoperiod treatments were selected: 1) 24 hours in light, 2) 12:12 hours light:darkness, and 3) 24 hours in darkness. A control bottle containing seawater without ^{13}C isotope was used in each treatment. At the end of the incubations the algae were washed with ~~abundant~~ seawater and rinsed with distilled water to remove the remains of the isotope that were not absorbed, and later frozen and lyophilized. Lyophilized samples (0.6 g) were depigmented twice in succession with methanol (100%) after that, low molecular weight carbohydrates were extracted in distilled water for 24 hours. Finally, the supernatant was frozen and lyophilized to be used in the NMR analysis.

^{13}C-Nuclear Magnetic Resonance Spectroscopy (NMR) analyses

To determine the incorporation of $NaH^{13}CO_3$ isotope in photosynthetic products, lyophilized samples (8 mg) were dissolved in 1 mL with 99.8% deuterium oxide (D_2O). The proton (^{13}C) spectra were recorded on a Varian/Agilent Premium Compact 600 NMR spectrometer (Palo Alto, CA, USA) at a frequency of 150.83 MHz using Sodium [3-trimethylsilyl 2,2′,3,3′-2-H4] propionate (TSP-d4) with internal reference to 0.00 ppm. All NMR spectra were recorded at room temperature using the following parameters: scans = 50,000; 13C-pulse width of 3.3 s, an acquisition time of 0.5 s, and a relaxation delay of 0.60 s.

Statistical analyses

To test the interactive effects of temperature, pH, and nutrient levels on P_{gross}, a three-way ANOVA (3 x 3 x 4) was performed. A Two-way ANOVA analyzed the effect ~~of~~ Q_{10} P_{gross} ~~on pH and nutrients~~. One-way ANOVA was applied to test for differences between different inhibitor assays whereas Newman-Keuls post-hoc multiple comparisons were used to test between treatments. All statistical tests and analyses were performed using the statistical package Statistica™ 7. Before analyses, homogeneity of variance (Bartlett) and normality test (Kolmogorov-Smirnov) were tested, and transformations were applied if necessary, to meet such criteria.

RESULTS

Photosynthetic responses to the interactive effect of temperature, pH, and nutrient ratios

The three-way ANOVA showed a significant interactive effect of temperature, pH, and nutrients on *H. scabra* gross photosynthesis, P_{gross} ($F_{12;\,216} = 4.57$, $p = 0.000$) (Fig. 1; Table S2). The highest P_{gross} values (1.83 mg O_2 g DW h^{-1}) were obtained at the highest nutrient ~~ratio~~ (10:1.0 µM) under elevated temperature (33°C) at a pH of 8.6 and in the control treatment (no nutrients added) at 33°C but, at the lowest pH (7.5) (P_{gross} = 1.78 mg O_2 g DW h^{-1}). In contrast, the lowest P_{gross} were observed in ~~all~~ treatments at the lowest temperature. In general, *H. scabra* photosynthetic rates were higher at the highest temperature tested regardless of the nutrient or pH ~~levels~~.

Effect of temperature on P_{gross} (Q_{10} P_{gross})

The two-way ANOVA showed a significant effect of ~~the~~ nutrient levels on ~~the~~ ~~P_{gross}~~, Q_{10} P_{gross} ($F_{2,\,54} = 6.721$, p = 0.002). This effect was more pronounced with the highest nutrient concentration (Q_{10} = 2.42) and decreased gradually as nutrient concentration decreased, from 1.75 to 0.75 in medium and low nutrient concentration, respectively. ~~The~~ pH and its interaction with nutrient levels did not show any significant effect on the Q_{10} calculated values (Table 1).

HCO_3^- Uptake

$\delta^{13}C$ value ~~found~~ in *H. scabra* was ~~of~~ -23.9‰ suggesting uptake of both HCO_3^- and CO_2, and the presence of a CCM. The carbonic anhydrase assays corroborate the ~~later~~ since the presence of EZ caused a significant inhibition (22.2%) of maximum photosynthesis rates P_{max} ($F_{3,\,80} = 18.674$, $p = 0.000$) whereas the combination of both inhibitors produced a similar effect to that found with EZ (Fig. 2).

Incorporation of ^{13}C into products of photosynthesis

^{13}C isotope labeling in *H. scabra* observed by signal multiplicity (coupling) also showed bicarbonate uptake, since ^{13}C isotope was incorporated into an amino acid akin to aspartate in the three photoperiod treatments analyzed. The incorporation in darkness indicates ~~a~~ non-photosynthetic ~~carboxylation by~~ β-carboxylation. Aspartate also appears in the three control treatments (simple decoupled signal) highlighting its abundance in the species (Fig. 3).

DISCUSSION

The results of our work support the hypothesis that a synergistic increase in pH, temperature, and nutrients enhances *H. scabra* photosynthesis. An increase in temperature could enhance P_{gross} at high pH if there ~~is~~ sufficient ~~availability of~~ nutrients. Environmental conditions of high seawater temperature (Robledo & Freile-Pelegrín, 2005; Rodríguez-Martínez et al., 2010), alkaline pH seawater likely because of the karstic origins of the Yucatán Peninsula (Cejudo et al., 2020) and, pulsatile nutrient enrichment due to the submarine groundwater discharge (Hernández-Terrones et al., 2015), are common in Quintana Roo coastal areas where *H scabra* and other *Halimeda* species colonize ~~successfully~~ shallow environments. Conversely, the interactive effect of decreasing pH (low and medium) with increases in temperature and nutrient enrichment kept P_{gross} below its potential ~~capacity and thus~~, potential deleterious effects on *H. scabra* performance are expected to occur under future scenarios of ocean acidification, global warming, and their complex interactions with nutrient enrichment due to the continuous coastal development in the area.

In agreement with our results with *H. scabra,* significant reductions in gross photosynthetic rates have been reported for *H. macroloba* and *H. cylindracea* when exposed to elevated CO_2 combined with elevated temperature, showing an additive negative effect (Sinutok et al., 2012). In contrast, for *H. incrassata*, *H. simulans*, and *H. opuntia* no significant effects in net photosynthesis were reported for the interactions ~~between~~ species, pH, and temperature (Campbell et al., 2016). While, in *H. opuntia* no interactive effect of CO_2 and nutrient enrichment on net photosynthesis was found (Hofmann et al., 2015).

~~Regardless the interpretation and its robustness,~~ the photosynthetic responses to the interactive effects of several environmental variables are complex since, in addition to the factors being evaluated, the physiological mechanisms could be responding to other interrelated processes that were not assessed during assays. For example, Campbell et al. (2016) found in three *Halimeda* species that photosynthesis was positively correlated to calcification rates and, an increase in temperature increased activity of both processes. In this context, processes with high carbon requirements such as calcification could indirectly stimulate photosynthesis (Carvalho & Eyre, 2017), generating protons that are used to facilitate the absorption of nutrients and bicarbonate (McConnaughey & Whelan, 1997). The reduction of NO_3^- to NH_4^+ is another process with high energy requirements (Ale, Mikkelsen & Meyer, 2011), and it is related to carbon fixation (Cabello-Pasini & Figueroa, 2005) so it is likely to be more plausible to affect photosynthesis rather than calcification since the latter appears to be more dependent on photosynthetic activity of many calcifying primary producers. ~~The~~ nutrient enrichment supported

a rapid increase in the physiological performance of *H. opuntia* (Teichberg, Fricke & Bischof, 2013). Therefore, the photosynthetic increase found with the nutrient addition (KNO_3:K_3PO_4) in our experiments could be the result of its effect on processes related to nutrient uptake and these, to photosynthesis. Moreover, it is also known that nutrient uptake rates increase with temperature increases (Harrison & Hurd, 2001), consequently, our results not only are a response to the interactive effect of environmental factors but also, the result of the direct and indirect response of other metabolic processes on photosynthesis.

Temperature is a significant factor controlling metabolic rates, including photosynthesis; increasing temperature increases ~~linearly~~ photosynthetic ~~rates~~ up to an optimum rate, beyond this thermal threshold rates, tend to decline (Bruno, Carr & O'Connor, 2015; Vásquez-Elizondo & Enríquez, 2016). It is generally accepted that Q_{10} values greater than 2 characterize an active nutrient absorption process across cell membranes, while $Q_{10} \sim 1$ values describe passive processes that are not greatly affected by temperature (Lobban & Harrison, 1994). According to this, our calculated Q_{10} P_{gross}, ~~fall between~~ the range of active nutrient absorption, expected by organisms living in highly illuminated habitats and with high elevated metabolic activity (Vásquez-Elizondo & Enríquez, 2016).

~~On the other hand, the carbon pathway~~ can influence the isotopic composition of organic matter. Values of $\delta^{13}C$ between -30 and -10% indicate both HCO_3^- and CO_2 active uptake and species who fixation fall within this range are classified as species with active CCM (Maberly, Raven & Johnston, 1992; Raven et al., 2002; Diaz-Pulido et al., 2016; Bender-Champ, Diaz-Pulido & Dove, 2017). ~~The~~ species with $\delta^{13}C$ signatures between –32% and –22% are considered as C3 plants while $\delta^{13}C$ between –16% and –10% are typical for C4 plants (Rautenberger et al., 2015; Valiela et al., 2018). Considering these ranges and the results obtained in this work, *H. scabra* could be classified as a C3 plant with a CCM that uses both, HCO_3^- and CO_2 as a resource of C_i for photosynthesis. The $\delta^{13}C$ values of *H. scabra* found within this study are in the range of those reported in other *Halimeda* species, such as *H. opuntia* (Zweng, Koch & Bowes, 2018), *H. tuna* (Duarte et al., 2018), and, *H. digitata* and *H. opuntia* (Vogel et al., 2015a).

~~Additionally to this,~~ the extracellular CA inhibitor (AZ), did not show any adverse effect ~~in~~ photosynthesis, ~~evidencing a direct entrance~~ of HCO_3^- while a significant reduction in P_{max} with EZ, ~~validated the~~ HCO_3^- uptake and the presence of a CCM (Badger et al., 1998) along with the role of internal CA (Badger & Price, 1994). The reduction of photosynthesis under the activity of EZ was only about 22.2% relative to control samples, likely because there was still enough CO_2 in the proximity of RuBisCO ~~allowing~~ to maintain photosynthesis ~~fully functional~~. The available CO_2 may come from the following alternatives: (1) as the result of a CCM; (2) ~~photosynthesis used~~ respiratory CO_2 (Borowitzka & Larkum, 1976); (3) CO_2 ~~was~~ supplied from ~~its accumulation~~ interutricular spaces, although this source is not sufficient to sustain photosynthesis (De Beer & Larkum, 2001); and (4) by CO_2 diffusion from both, the external medium and the intercellular space (ICS) (Borowitzka & Larkum, ~~1976.~~ All the previous explanations could maintain RuBisCO CO_2 saturated and minimize photosynthetic losses after inhibiting intracellular CA. This suggests that inorganic carbon supply for photosynthesis in *H.*

scabra does not depend entirely on the activity of the CA´s, and might be maintained by several ways, which may be advantageous during adverse conditions. Bicarbonate (HCO_3^-) uptake has also been found in *H. discoidea*, *H. macroloba*, and *H. tuna* (Borowitzka & Larkum, 1976), while the lack of extracellular CA has been found in *H. discoidea* (De Beer & Larkum 2001) and *H. cuneata* f. *digitate* (Hofmann et al., 2015a).

Some *Halimeda* species possess CCM and use bicarbonate as an alternate source of inorganic carbon for photosynthesis (Borowitzka & Larkum, 1976; Price et al., 2011). Therefore, this ability may also be responsible for P_{gross} enhancement under elevated temperature observed in this study in *H. scabra* through a decrease in photorespiration (Ogren, 1984). According to Giordano, Beardall & Raven (2005), the number of resources that a cell invests in acquiring carbon through a CCM is likely to be coupled with the availability of nutrients. This could also explain the increase in P_{gross} observed at the highest nutrient ratio since most CCM´s require the *de novo* synthesis of specific proteins, which represents a demand for cellular nitrogen (Giordano, Beardall & Raven, 2005). The low oxygen production observed at low pH (under high nutrient concentrations) in *H. scabra* could be a delay in the induction of the CCM relying on passive diffusion of CO_2 alone, thus leading to reduced efficiency of carbon assimilation (Price et al., 2011; Cornwall et al., 2012; Meyer et al., 2016).

In this study, *H. scabra* incorporated ^{13}C isotope into aspartate in the three photoperiod treatments demonstrating $NaH^{13}CO_3$ assimilation photosynthetic and non-photosynthetic. Moreover, the incorporation of ^{13}C isotope in aspartate in 24-hour darkness treatment indicates β-carboxylation which facilitates a metabolic alternative to inorganic carbon carboxylation resulting in an important contribution to CCMs (Raven & Osmond, 1992; Enríquez & Rodríguez-Román, 2006). In general, β-carboxylation has multiple functions for algal metabolism such as providing essential compounds for growth that cannot be produced photosynthetically (Falkowski & Raven, 2007). Carbon fixation of these compounds can be done in both light and darkness (Axelsson, 1988), and it is generally less than 5% of maximum photosynthesis (Cabello-Pasini & Alberte, 1997). In marine algae, the end products of this carbon fixation independent of light are typically organic compounds and amino acids rather than triose sugars generated during photosynthesis (Cabello-Pasini & Alberte, 1997). Although the $\delta^{13}C$ values found in *H. scabra* suggest a C3 pathway, the abundance of aspartate in control and experimental treatments might suggest the existence of a C4 pathway. In C4 plants, the C4 acids malate and aspartate are the major initial photosynthetic products, these products are rapidly decarboxylated releasing CO_2 for its refixation by RuBisCO functioning as photosynthetic intermediates (Holaday & Bowes, 1980). In this sense, a C4 mechanism could explain the increase in P_{gross} under all of our high-end treatments (temperature, pH, and nutrient ratio), as well as the insensitivity of the photosynthetic response of the alga to AZ and, its low inhibition in the presence of EZ. C4 plants have an active CCM, which is mainly related to the efficient use of HCO_3^- through an initial carboxylation reaction by a Phosphoenolpyruvate enzyme (Badger & Price, 1994). C4 mechanisms have been reported in some Chlorophyta, including the semi-calcified *Udotea flabellum,* which shows an initial carboxylation by

phosphoenolpyruvate carboxykinase enzyme (Reiskind, Seamon & Bowes, 1988), whereas in *Ulva prolifera* evidence of both C3 and C4 pathway has been found (Xu et al., 2012).

CONCLUSIONS

H. scabra uses both ~~sources of Ci~~ for photosynthesis and it seems to have different mechanisms for ~~its~~ acquisition incorporating bicarbonate through ~~the~~ photosynthetic and non-photosynthetic pathways. ~~The evidence found in this work~~ suggest the presence of both C3 and C4 pathways, ~~the latter rely~~ on β-carboxylation. These strategies give *H. scabra* physiological plasticity to acclimate to possible environmental changes in the short term. Our study strongly suggests that *H. scabra* acclimatizes better to environmental conditions ~~with interactive effect at~~ high pH and high temperature with enough nutrient enrichment. Although ~~this~~ could exacerbate the presence of epiphytes and opportunistic algae, the ~~entrance~~ of pulsatile nutrients likely plays a role in maintaining ~~this balance~~ by enhancing algal photosynthetic performance. Such conditions are typical in the Yucatan peninsula coast where ~~the~~ ~~algae~~ grow in abundance. Opposite interactive conditions of decreasing pH in combination with increases in temperature and nutrient availability, could keep photosynthesis at a sub-optimal level which has strong ecological implications due to the ~~decline of their~~ abundance and the ~~consequences of it over~~ sediment production and carbon balance in coral reefs where these algae thrive.

ACKNOWLEDGMENTS

The authors thank Dr. D. Valdés, V. Avila Velázquez, and C. Chávez for their help in setting up the experiments. To Dr. E. Serviere-Zaragoza for the tissue isotopic analysis. The first author is grateful to Conacyt Ph.D. scholarship N° 704918/593231 and Fomix-Conacyt project FOMIX-YUC-2014-C17-247043.

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581

582 **Figure legends**

583 Figure 1. Interactive effect of temperature, pH, and nutrients on the gross photosynthesis rate
584 (P_{gross}) of *H. scabra*. ~~Three-way ANOVA.~~ Symbols represent the mean and error bars 0.95
585 confidence intervals.

586 Figure 2. Comparison of the effect of two carbonic anhydrase inhibitors on P_{max} percentage. Error
587 bars represent confidence intervals at 0.95.

588 Figure 3. NMR Spectra of $NaH^{13}CO_3$ incorporation into photosynthetic products of *H. scabra* in
589 different light and darkness treatments. A) 24 h at saturation irradiances; B) 12 hours under light
590 saturation and 12h in darkness and, C) 24 h in darkness. * Indicate consistent signals for aspartate
591 in control treatment; ** Indicate ^{13}C enrichment (multiple coupling). Letter **a** ~~indicate~~ control,
592 Letter **b** ~~indicate~~ treatment.

Figure 1

Interactive effect of temperature, pH, and nutrients on the gross photosynthesis rate (P_{gross}) of *H. scabra*. ~~Three way ANOVA.~~ Symbols represent the mean and error bars 0.95 confidence intervals.

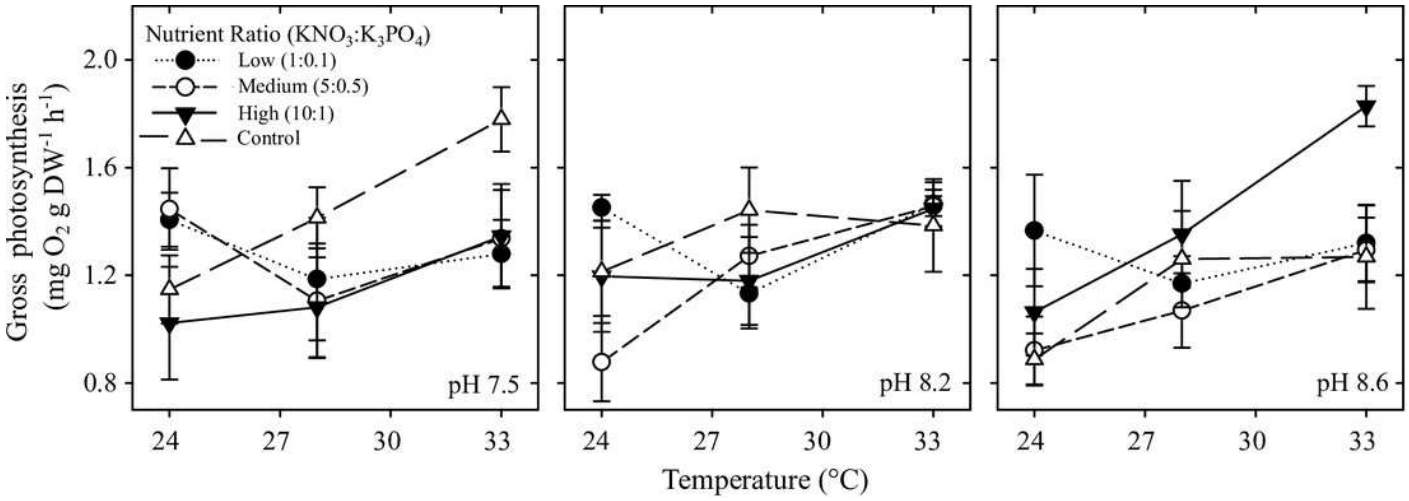

Figure 2

Comparison of the effect of two carbonic anhydrase inhibitors on P_{max} percentage. Error bars represent confidence intervals at 0.95.

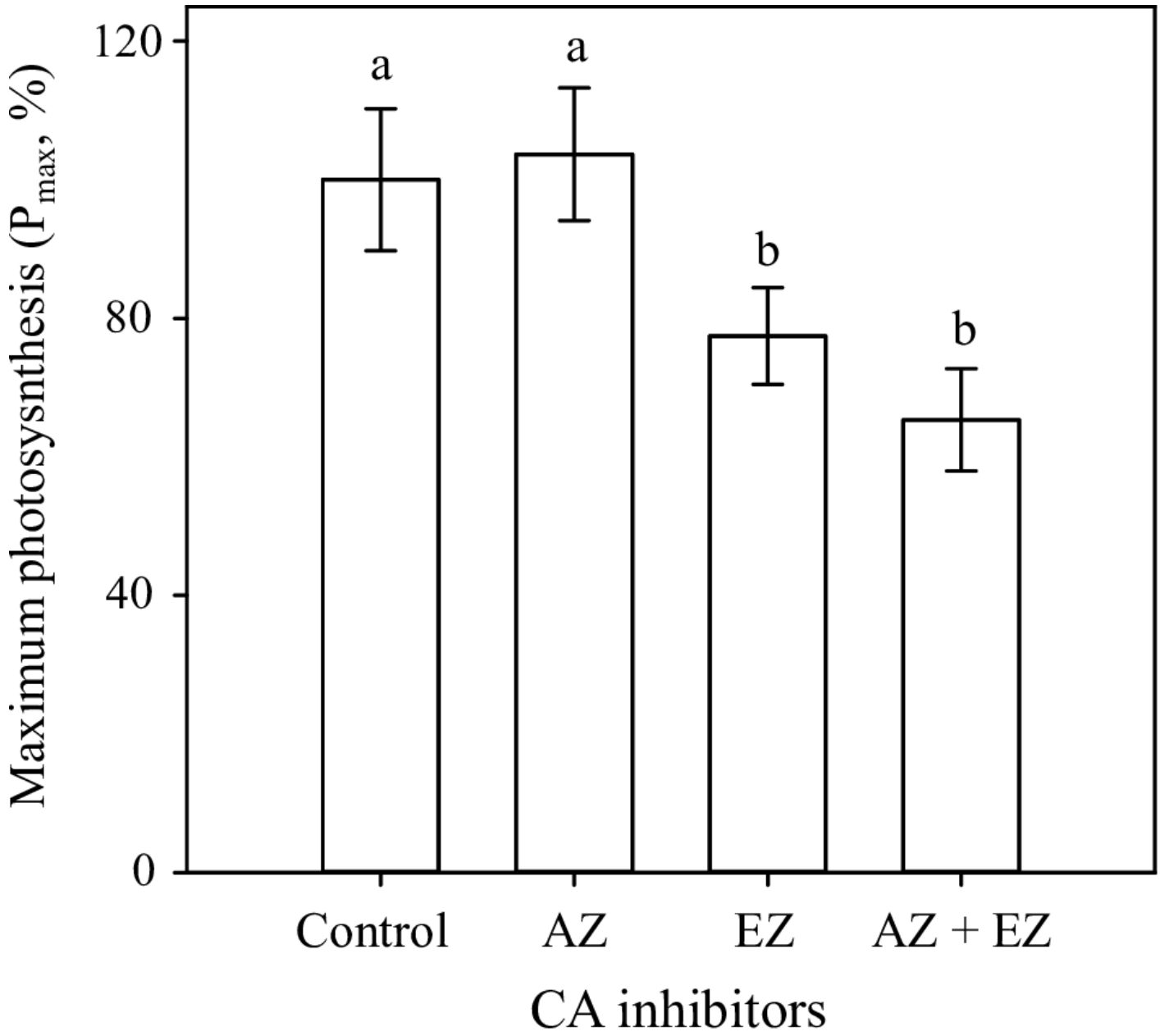

Figure 3

NMR Spectra of $NaH^{13}CO_3$ incorporation into photosynthetic products of *H. scabra* in different light and darkness treatments. A) 24 h at saturation irradiances; B) 12 hours under light saturation and 12h in darkness and, C) 24 h in

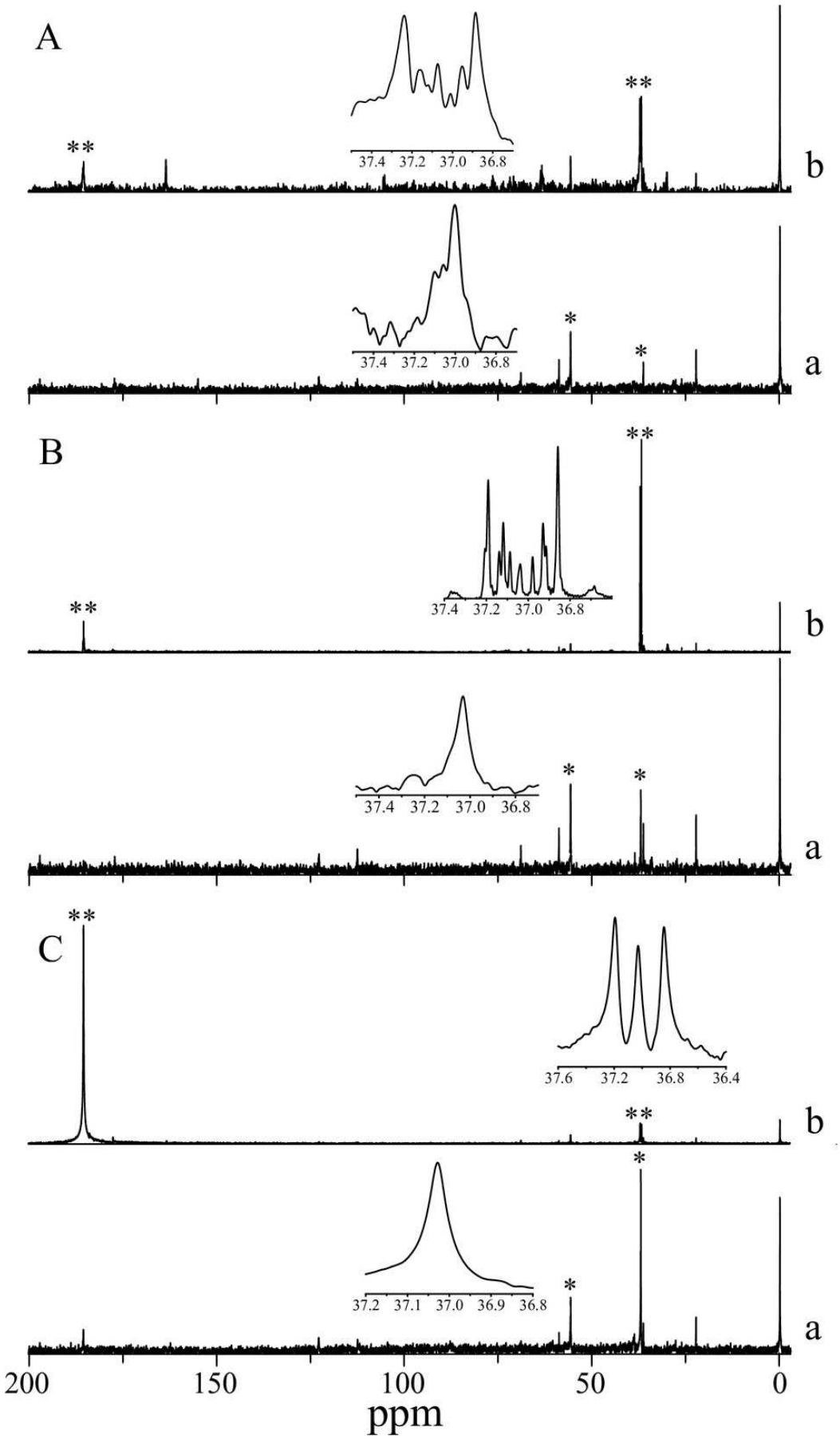
A
B
C
a
b
**
*
37.4 37.2 37.0 36.8
37.6 37.2 36.8 36.4
37.2 37.1 37.0 36.9 36.8
200 150 100 50 0
ppm

Table 1(on next page)

Effect of temperature on P_{gross} (Q_{10}) in three nutrient concentrations (Two Way-ANOVA).

Table 1:

Effect of temperature on P_{gross} (Q_{10}) in three nutrient concentrations (Two Way-ANOVA).

	Mean Q_{10}	SS	DF	MS	F	p
Nutrient ratio		10.839	2	5.419	6.721	0.002 ***
1.0:0.5	0.75[b]					
5.0:0.5	1.75[a]					
10.0:1.0	2.42[a]					
pH		3.137	2	1.568	1.945	0.152 ns
nutrients*pH		6.111	4	1.528	1.895	0.125 ns
Error		43.543	54	0.806		

ns: not significant; *** significant