Photosynthesis and light-dependent proton pumps increase boundary layer pH in tropical macroalgae: A proposed mechanism to sustain calcification under ocean acidification

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\section*{ABSTRACT}

Ocean acidification (OA) projections predict ocean pH to decline between 0.2 and 0.4 by 2100 with potential negative consequences for marine calcifiers without acclimation or adaption strategies to accommodate greater $[\text{H}^+]$ in seawater. Biotic control of calcified reef macroalgae thalli surface diffusive boundary layer (DBL) chemistry may overcome low pH in seawater as one strategy to accommodate OA conditions. To investigate this strategy, we examined surface DBL O$_2$ and pH dynamics in five calcifying macroalgae (\textit{Halimeda}, \textit{Udotea}, \textit{Jania}, \textit{Neogoniolithon}, crustose coralline algae [CCA]) from the Florida Reef Tract under ambient (8.1) and low (7.65) pH using microsensors (100 μm) at the thalli surface in a flow-through flume. The role of photosynthesis and photosystem II (PSII)-independent proton pumps in controlling DBL pH were examined. Four of the five macroalgae exhibited a strong positive linear relationship between O$_2$ production and increasing pH in the first 15–30 s of irradiance. Once a quasi-steady-state O$_2$ concentration was reached (300 s), all species had DBL pH that were higher (0.02–0.32) than bulk seawater. The DBL pH increase was greatest at low pH and dependent on PSII. Some evidence was found for a light-dependent, but PSII-independent, proton pump. High DBL Δ pH upon illumination was likely in response to carbon concentrating mechanisms (CCMs) for photosynthesis. CCMs may be a HCO$_3^−$–H$^+$ symport, OH$^−$ antiport or other DIC transport system, accompanied by proton efflux. HCO$_3^−$ dehydration by external carbonic anhydrase (CAext) also produces OH$^−$ that can neutralize H$^+$ in the DBL. CO$_2$ or HCO$_3^−$ uptake for photosynthesis may also engage H$^+$/OH$^−$ fluxes as part of intracellular acid-base regulation changing DBL pH. A higher Δ pH within the DBL at low pH could be accounted for by greater CO$_2$ diffusion and/or lower efficiencies in exporting cellular H$^+$ across a lower concentration gradient, and/or a more efficient removal of H$^+$ by CAext-driven dehydration of HCO$_3^−$. In the dark, Δ pH was less than in the light as these dynamics were primarily due to photosynthesis. We present a conceptual model of inorganic carbon uptake and ion transport pathways, as well as other processes associated with photosynthesis that drive DBL Δ pH and sustain tropical macroagal calcification in the light under OA. In the dark, unless PSII-independent proton pumps are present, which do not appear to be ubiquitous amongst species, acidification processes likely dominate, resulting in CaCO$_3$ net dissolution, particularly under OA conditions.

1. Introduction

Ocean acidification (OA) projections (RCP2.6 and RCP8.5 AR5; IPCC et al., 2013), predict surface ocean pH to decline between 0.2 and 0.4 by 2100 (Gehlen et al., 2014; Hartin et al., 2016; IPCC, 2013). This results from ocean sequestration of ~30% of anthropogenically released CO$_2$ (Sabine et al., 2004) which is predicted to triple under RCP8.5 scenario to ~28 Pg C yr$^{-1}$ by 2100 (Riahi et al., 2011). A 0.1 decrease in pH already observed in the oceans since the industrial revolution (Caldeira and Wickett, 2003) equates to a 30% increase in hydrogen ion $[\text{H}^+]$ concentration. Ocean dissolved inorganic carbon (DIC) chemistry is influenced by changes in pH due to its regulatory effect on the carbonate equilibria. In response to a pH decrease of 0.4, the carbonate equilibria under OA leads to a ~200% increase in CO$_2$ and a ~25% increase in bicarbonate (HCO$_3^−$), while lowering carbonate (CO$_3^{2−}$) by ~50–60% (Fabry et al., 2008; Koch et al., 2013). The decline in CO$_3^{2−}$ lowers the saturation state (Ω) of carbonate minerals of calcite and aragonite (~60%). The high concentration of Ca$^{2+}$ in
seawater (~10,000 μmol kg⁻¹) is considered non-limiting for calcification, thus calcium carbonate production is chemically controlled by the concentration (100–200 μmol kg⁻¹) of CO₂⁻ (Millero et al., 2007; Millero et al., 2006). The reduction in CO₂⁻ under OA is a major concern because of the potential negative consequences for calcification in marine organisms (Doney et al., 2012; Doney et al., 2009; Fabry et al., 2008; Gattuso et al., 2015; Kroeker et al., 2010; Orr et al., 2005; Sabine et al., 2004). Calcification rates have been shown to be correlated to seawater ΩCaCO₃ and studies indicate higher calcification rates under elevated [CO₂⁻] (Anthony et al., 2008; Gao et al., 1993; Gattuso et al., 1999; Hoegh-Guldberg et al., 2007; Langdon et al., 2000).

While the saturation state of CaCO₃ [CO₂⁻], and [Ga²⁺] are recognized as important for calcifiers (Comeau et al., 2012; Waldbusser et al., 2016), some studies show no relationship between calcification rates and ΩCaCO₃ in the bulk seawater and inconsistent calcification responses by a diversity of marine organisms to lower ΩCaCO₃ (Comeau et al., 2018; Comeau et al., 2016; Dutra et al., 2015; Hendriks et al., 2010; McDonald et al., 2009; Peach et al., 2017b; Rodolfo-Metalpa et al., 2011; Shamberger et al., 2014). These results led Jokiel (Jokiel, 2013, 2011) and others (Bach, 2015; Cyronak et al., 2015) to re-evaluate the importance of ΩCaCO₃ and put forth an alternative hypothesis. They suggest that limitations to calcification are a consequence of a buildup of [H⁺] at the site of calcification due to an inability to expel protons from the calcifying space into the bulk seawater under OA conditions. However, this theory is based primarily on corals where mineralization is below an epithelial layer separating the calcification site from seawater, while marine macroalgae calcify in their cell walls and extracellularly in close proximity to bulk seawater. In macroalgae, the diffusive boundary layer (DBL) microchemistry, driven by cellular metabolic processes, membrane transport systems and/or thalli morphologies control the calcification site exposure to bulk seawater chemistry.

Therefore, the question for macroalgae is can they control their DBL chemistry through proton pumps and employ mechanisms to take up CO₂ and HCO₃⁻ that raise DBL pH and maintain calcification under OA. Relatively new applications of the boron isotopic composition provide evidence that marine calcifiers, including corals and calcifying algae, have the capacity to maintain a high pH in their calcifying fluid (pHcf) even under OA conditions (pH 7.64) where [CO₂⁻] and ΩCaCO₃ of the bulk seawater are low (Comeau et al., 2018; Cornwall et al., 2017a; Donald et al., 2017; McCulloch et al., 2012). Understanding these mechanisms are particularly critical in calcifying autotrophs that require HCO₃⁻ and/or CO₂ for photosynthesis (Hurd et al., 2011; Koch et al., 2013), a major driver of high calcification rates (De Beer and Larkum, 2001), and in some species initiates calcification (Wizemann et al., 2014). For marine calcifying macroalgae, the increase in HCO₃⁻ and CO₂ under OA promote DIC availability for photosynthesis (Cornwall et al., 2017b; Koch et al., 2013; Zweng et al., 2018). Greater [DIC] may result in higher rates of calcification or compensate for OA effects, as photosynthesis and calcification may be in competition for DIC or control the DIC/H⁺ ratios affecting calcification. Further, the majority of marine macroautotrophs are restricted to energetics from photosynthesis, while corals and other heterotrophs can acquire energy to maintain a high pHcf through feeding (McCulloch et al., 2012).

Several recent studies examining the thallus boundary conditions of marine macroalgal calcifiers indicate a high degree of biotic control to elevate pH in the light at the thalli surface (Cornwall et al., 2014, 2013; Hofmann et al., 2018, 2016; Hurd et al., 2011) and in their calcifying fluids (Comeau et al., 2018; Cornwall et al., 2017a; Donald et al., 2017). As pH increases at the thalli surface across species and calcification location, a general model may emerge on the role of biotic pH control on calcification with closer examination of a diversity of calcifying macroalgae. In the present study, five tropical reef macroalgae were examined for their ability to regulate thallus surface DBL pH when bulk seawater pH was reduced from ambient conditions (8.1) to those projected for the year 2100. Microsensors were used to establish pH and O₂ dynamics to discern potential mechanisms controlling DBL microchemistry under OA conditions and how these DIC and ion fluxes may subsequently influence calcification. The DBL pH dependence on light, photosynthesis, and light-dependent photosystem II-independent proton pumps were also examined for each species. We present a general conceptual model to formulate our hypothesis of how DIC pathways and biotic control of pH could shift under OA and subsequently affect calcification.

2. Methods

2.1. Algal collection

Five dominant calcified Florida Reef Tract macroalgae (Fig. 1) were collected from a patch reef (~4 m depth) offshore of Looe Key Reef and offshore of Big Pine Key, FL (24° 37.233′ N, 81° 22.247′ W; Oct 2017 – Jan 2018); hereafter referred to by genus names. The five tropical reef calcifiers in this study include: three high-Mg calcite rhodophytes (Neogoniolithon strictum and Jania adhaerens [branching], and crustose coralline algae (CCA) [prostrate]) and two aragonite filamentous chlorophytes (Halimeda scabra [segmented, branching plates] and Udotea luna [fan shaped]). CCA was collected following establishment onto settlement plates (2 × 6 cm) over a 4-month period. All species and settlement plates were transported back to the Florida Atlantic University (FAU) lab in an aerated cooler and experiments run within two weeks. During collections, irradiance was determined using an underwater 4π spherical quantum sensor (LI-COR). Temperature, salinity and O₂ of the overlying water were measured using a multisensor probe (600 XLM, YSI Inc.). Surface water pH was also measured from discrete samples (Orion A211, 8302BNUMD) following calibration with NBS standards pH 7.00 and 10.00 and corrected with a standard TRIS buffer (Dickson Lab, Scripps Institute of Oceanography). Seawater samples (150 mL) from the collection site were stored cooled (4 °C) in the dark and total alkalinity (TA) determined by titration within 48 h.

Algae were held in 9L aquaria with seawater (35 psu) collected from FAU’s marine lab (Atlantic Ocean, Boca Raton, FL) in a water-bath
maintained at 28 °C and illuminated (~300 μmol photons m$^{-2}$s$^{-1}$) under a 12 h day/night cycle. Aquaria seawater was replenished (75%) every other day to maintain salinity and provide nutrients with pH and TA similar to collection site (8.09 and 2298 μmol kg$^{-1}$ sw, respectively); aquaria were continuously aerated.

2.2. Microsensors and experimental setup

Microsensor measurements were conducted in a flow-through flume system (Fig. 1a) using filtered (0.45 μm) seawater from the FAU marine lab maintained at 28 °C. Flume seawater pH$_{NBS}$ was continuously measured (1 min) in the flume (Orion A211, 83028BNUMD) after calibration and corrected with a TRIS buffer (Dickson Lab, Scripps Institute of Oceanography). The O$_2$ was measured using an optical sensor (Orion A329) at the beginning and end of each microsensor experiment. During each run, TA was determined on flume seawater and used to calculate carbonate speciation with CO2SYS (Pierrot et al., 2006). Carbonate chemistry parameters were calculated using the pH treatments and average Florida Reef tract temperature (28 °C) and salinity (35 psu) used for microsensor runs. The pCO$_2$ treatment was 1200 μatm for low pH (7.66 ± 0.02) and 360 ± 28 μatm for ambient pH (8.12 ± 0.03) controls (Table S1). The calcium carbonate saturation state (Ω$_{CaArag}$) of aragonite and calcite were 2–3 times higher in the ambient controls (6.0/3.8) relative to low pH treatments (Table S1). The ΔpH during the runs in the bulk seawater from the initial to end of experiments was 0.01 to 0.03.

Within the flume system, macroalgae were secured approximately 10 cm from the chamber bottom in the center of the flume to receive laminar flow (Fig. 1a–e). Water flow was provided by a submersible 300 l hr$^{-1}$ pump in the sump tank. Seawater was pumped from the sump to the flume where seawater passed through a perforated barrier in order to create laminar flow. Laminar flow and velocity were previously determined by injecting dye into the sump tank and recording flow in the flume. The flume was illuminated by a full-spectrum LED light (Kessil, A360W E-Series Tuna Sun) set at 500 μmol photons m$^{-2}$s$^{-1}$ during light runs. The O$_2$ and pH microsensors were positioned within 200 μm of each other on the thalli surface using a manual dual-head micromanipulator arm (MM33–2, Unisense) and measurements were recorded simultaneously every second. Thalli surface O$_2$ and pH dynamics were determined using miniaturized amperometric sensors detecting oxidation-reduction signals (pA range) with rapid response times (0.3 s) and recorded with a highly sensitive picoammeter (Unisense UnderWater Meter System, Denmark). The microsensors (100 μm) are connected to a 4-channel underwater recorder equipped with amplifiers (ISA) that provide a high-quality signal with low background interference. Prior to measurements, the sensors are polarized and calibrated. The O$_2$ microsensor (OX-100, Unisense Inc.) was calibrated using aerated seawater at 100% saturation and an anoxia solution used for 0% saturation (0.1 M sodium ascorbate and NaOH; ~2 g sodium ascorbate in 100 mL of 0.1 M NaOH). The pH microsensor (pH-100, Unisense Inc.) was calibrated using NBS buffers (7.00 and 10.00) and corrected using a TRIS buffer (Dickson Lab, Scripps Institute of Oceanography).

2.3. pH and O$_2$ dynamic experiments

After samples were secured in the flume and microsensors moved into position, light:dark cycle runs proceeded. There was an initial 10-min dark pretreatment period followed by 3 continuous light:dark cycles run at 5 min intervals. The first was conducted at a pH of ~8.1 (ambient). The flume seawater or bulk water pH was then lowered to ~7.65 (low pH) by the addition of acidified seawater produced by bubbling with pure CO$_2$ (Riebesell and Gattuso, 2011) and added in 20 ml aliquots to the water bath in line with the flume system. Once bulk seawater pH and thalli microsensor pH stabilized, the algae was again pretreated for 10 min in the dark, followed by another light:dark cycle. At the end of the low pH light:dark cycle, the bulk seawater pH was adjusted to pH 8.1 by adding 0.05 M NaOH in 2 ml aliquots to the water bath and inhibitor experiments conducted. Test runs showed no difference in pH and O$_2$ surface dynamics after the addition of NaOH to re-establish ambient pH conditions, and adjustments were only performed before the inhibitor experiment.

To determine the role of photosynthesis in thallus surface pH dynamics, a photosystem II inhibitor, herbicide 3-(3,4-Dichlorophenyl)-1,1-dimethyleurea (DCMU), was amended to the flume system. The concentration to be used for each run was established by the minimum level needed to inhibit O$_2$ production at the thalli surface of the species examined (~2–4 μM DCMU). Although DCMU has been shown to elevate respiration in some macroalgae, no change in [O$_2$] was found with DCMU for any species or run (Table S2). Diminished O$_2$ concentrations at the thalli surface and no O$_2$ change under irradiance was used to confirm photosynthesis inhibition before light:dark cycles commenced. The light:dark cycle runs were conducted at pH 8.1 and 7.65 as described above.

3. Statistical analysis

To compare amongst treatments and define O$_2$ and pH dynamics, t-tests and regression analyses were performed (SigmaPlot 13.0, Systat Software Inc.). A t-test was used to examine O$_2$ and pH changes at the thalli surface in response to light (light-dark) and pH (ambient-low) changes. For these comparisons, the average of the first and last 10 s of the dynamic runs (n = 3) were calculated and the differences used in the analysis. The same approach was used for the runs with the photosynthetic inhibitor (DCMU). Thalli surface pH was also compared to the bulk seawater pH in the light and dark, as well as in the ambient and low pH treatments. A Mann-Whitney U non-parametric test was used if data were non-normal or variances were not homogeneous. Linear regression analysis was used to establish the relationship (slope, R$^2$) between photosynthesis (O$_2$ production) and pH change using the initial 30 s of the light run. The dynamics in the light and dark across the entire 300 s dynamic run were described using linear and non-linear regression analysis.

4. Results

4.1. Microsensor runs

Three independent microsensor runs using different individuals produced a range of thallus surface O$_2$ concentrations after 300 s for each species ranging from 73 to 852 μM of O$_2$ (Table 1). However, the replicates within each microsensor run were highly repeatable with low variance across three sequential light:dark cycles and pH treatments (Fig. 2). Thus, pH and light-dark comparisons of O$_2$ and pH dynamics were examined within each run (Tables 1–3), and details of the dynamics are presented in graphs for light-dark incubations in microsensor run one (Figs. 2–4) with additional runs presented in the supplement (Fig. S1, S2).

4.2. Dark-light O$_2$ dynamics

The change in O$_2$ from dark to light in ambient, compared to low pH, was not significant for most of the species across all three microsensor runs (Fig. 2; Table 1) with the exception of CCA and *Halimeda* in runs one and two. During these runs, the change in O$_2$ in response to light was always greatest at low pH. Although these results indicate a potential increase in photosynthesis with greater CO$_2$ availability at low pH, the percent increase was only 21 and 23% for CCA and *Halimeda*, respectively. The consistency of O$_2$ dynamic changes from light to dark incubations is clearly seen in the replicate incubations in run one where the O$_2$ scale was equivalent for both ambient and low pH runs (Fig. 2) and in runs two and three (Table 1). In contrast to relatively high [O$_2$]...
Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Microsensor Run 1</th>
<th>Microsensor Run 2</th>
<th>Microsensor Run 3</th>
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<tbody>
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<td></td>
<td>Ambient (μM)</td>
<td>Low (μM)</td>
<td>Ambient (μM)</td>
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<tr>
<td>Neo</td>
<td>442 ± 9</td>
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<td>Jan</td>
<td>328 ± 46</td>
<td>304 ± 48</td>
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<tr>
<td>CCA</td>
<td>236 ± 12*</td>
<td>265 ± 8*</td>
<td>137 ± 9*</td>
</tr>
<tr>
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<td>559 ± 26*</td>
<td>608 ± 14*</td>
<td>139 ± 10*</td>
</tr>
<tr>
<td>Udot</td>
<td>83 ± 4</td>
<td>71 ± 7</td>
<td>73 ± 3</td>
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4.3. Dark-light pH dynamics

The initial (15–30 s) light-induced increase in pH at the thalli surface was highly correlated with O₂ production (R² 0.86–0.98) for all species (Fig. 3a–d) with the exception of CCA (Fig. 2c). The slope of the initial pH response was relatively similar under ambient and low pH (Fig. 3a–d), but was an order of magnitude greater for Halimeda compared to the other species (Fig. 3c). While CCA had an immediate O₂ flux response to light, there was a lag in pH increase (Fig. 2c).

In contrast to short-term (15–30 s) pH dynamics, over the entire 300 s light incubation, Δ pH was dependent on the quasi-steady-state O₂ concentration after 300 s in the light (Fig. 4c).

The pH dynamics in the dark were similar for Neogoniolithon, Jania and Udotea which all followed a hyperbolic decay model with relatively similar slopes at low and ambient pH (Fig. 4a, b, e; Table 4). The non-linear models of pH decline in the dark were similar between pH treatments compared to more diverse responses in the light. Halimeda showed a rapid decline in pH during the dark incubation for the first 100 s, followed by an increase in pH, fitting an exponential decay function with a linear combination (Fig. 4d, Table 4). Consistent with the light dynamics, CCA had a slow 2-phase decline in pH over time, but still fit the hyperbolic decay model.

Following treatment with DCMU, Δ pH from dark to light was lowered significantly. There was also no difference in the Δ pH between ambient and low pH. Thus, the dark/light changes in pH at the thalli surface was dependent on photosynthesis for most species. The greater Δ pH under OA conditions was also linked to photosynthesis, as no significant differences in Δ pH at low pH were observed after treatment with DCMU. Only 2 species (Neogoniolithon, Halimeda) continued to exhibit pH dynamics during the dark-light cycles with DCMU, albeit the magnitude of change was significantly reduced. Both at ambient and low pH, Neogoniolithon had the capacity to raise pH ~0.03 to 0.04 units in the light following a 300 s dark incubation (Fig. 5). Halimeda showed a reverse response to Neogoniolithon, increasing pH ~0.03 to 0.04 units in the dark, and lowering pH at the thalli surface in the light (Fig. 5). This increase in pH during the dark incubation for Halimeda is consistent with the 300 s dark incubation that showed an exponential decrease in pH followed by a slower linear rise over time (Fig. 4d).

4.4. Thalli surface vs bulk seawater pH

In addition to ~33% greater Δ pH between dark-light cycles at low compared to ambient pH, we observed a 133% increase in pH at the thalli surface relative to the bulk seawater pH at low compared to ambient pH in the light. Averaging results from all five species, there was a higher pH at the thalli surfaces relative to the bulk seawater at low (average = 0.14) compared to ambient (average = 0.06) pH in the light, and significant differences detected for at least two out of three runs for each species (Table 3). Even in the dark, the pH was higher at the thalli surface relative to bulk seawater in the light and significant differences were observed for all species (Table 3).

5. Discussion

Four out of five tropical calcifying reef macroalgae exhibited a strong linear relationship between O₂ production and increasing pH in the first 15–30 s of the runs, with two of the rhodophyte species (Neogoniolithon and Jania) and Halimeda exhibiting an R² ≥ 95. Once a quasi-steady-state O₂ concentration was reached after 300 s in the light,
pH at the thalli surfaces was always greater (0.02–0.32) than in the overlying bulk seawater for all species. This increase in pH at the thalli surface was primarily dependent on photosynthesis, as shown by no significant Δ pH between ambient and low pH treatments when PSII was inhibited with DCMU. A metabolically-driven increase in pH within the diffusive boundary layer (DBL) in the light has been observed in fleshy (Noisette and Hurd, 2018) and calcifying (Cornwall et al., 2015, 2013; De Beer and Larkum, 2003; Hofmann et al., 2018, 2016; Hurd et al., 2011) macroalgae. Encrusting coralline algae (Sporolithon durum) raised pH in the light 0.88 pH units from 8.0 pH after 1 h under a flow rate of 1.5 cm s⁻¹ (Hurd et al., 2011), similar to the flow in our experiments (~2–3 cm s⁻¹). Cornwall et al. (2013) attributed a pH increase of ~0.35 in the light within the DBL of an erect coralline and encrusting algal consortium at flow rates < 4 cm s⁻¹ to photosynthesis. Photosynthetic inhibitors (AZ, DIDS) lowered a 0.3 to 0.7 pH increase by ~30% at the surface of a crustose coralline algae (CCA) (Hofmann et al., 2016). These data, and those showing a correspondence between increasing CO₃²⁻ and pH at algal surfaces using microsensors (Chrachri et al., 2018; Hofmann et al., 2018), infer that photosynthesis drives pH and DIC microchemistry within the DBL by the exchange of CO₂ and ions (H⁺, OH⁻, HCO₃⁻) across the plasmalemma (Borowitzka, 1981; Borowitzka and Larkum, 1987; Gao et al., 1993). HCO₃⁻ uptake via a H⁺ symport (or OH⁻ antiport) or other H⁺ transport mechanism (e.g., Ca²⁺:H⁺ antiport, Na⁺:H⁺ antiport) and/or external carbonic

Fig. 2. Complete dark-light cycles (n = 3) showing O₂ (dark circles) and pH (grey circles) thalli surface dynamics for each species (a = Neogoniolithon, b = Jania, c = CCA, d = Halimeda, e = Udotea). Panels on left show dynamics at ambient pH, while those on the right are at low pH; note different right axis scale for pH treatments. The bulk seawater pH is also shown (grey dotted line) in each figure. Each cycle starts with lights (500 μmol photons m⁻² s⁻¹) on for 300 s followed by a 300 s dark period.
anhydrase enzyme (CAext)-mediated dehydration of HCO₃⁻ to CO₂ and OH⁻ would raise the pH at the thallus surface. HCO₃⁻ dehydrogenation by CAext to CO₂, followed by diffusive flux into the cell for photosynthesis, was predicted by Chrachri et al. (2018) to drive a rapid pH change in the DBL of large diatoms. Active HCO₃⁻ uptake and CO₂ diffusion mediated by CAext may be not be exclusive. A H⁺ efflux across the plasmalemma, catalyzed by a H⁺-ATPase in support of a HCO₃⁻/H⁺ symport, would create microzones of low pH that would enhance external dehydration of HCO₃⁻. While suspected to occur, these low pH microzones at the thalli surface have not been identified (Raven and Hurd, 2012). Although the CAext enzyme speeds up the equilibria reactions between HCO₃⁻ and CO₂, an initial mechanism is required to initiate a shift in the carbonate equilibria. Support for additional HCO₃⁻ transport mechanisms have been evidenced by incomplete reduction in photosynthetic rates (O₂ flux) using CAext inhibitors in both macroalgae (Hofmann et al., 2016) and phytoplankton (Chrachri et al., 2018). In addition to direct effects, intracellular acid-base regulation in response to photosynthesis can generate H+/OH⁻ fluxes across the plasmalemma shifting DBL pH. Regardless of the DIC-uptake or ion transport mechanism, photosynthesis promotes Δ pH which has been attributed to maximum rates of calcification in macroalgae and corals (De Beer and Larkum, 2001; Gattuso et al., 1999; Martin et al., 2013). Thus, an

### Table 2

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### Table 3

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</tbody>
</table>
DBL pH with photosynthesis will be compromised at low pH under OA. An important question is whether or not photosynthesis and increase of the O2 concentration at the thalli surface in the light was significantly higher at low pH relative to bulk seawater, the increased Δ pH was always positive, and the pH change was faster than predicted based on chemical equilibria (CO2SYS RF change with pH). Enhanced Δ pH under OA conditions was arrested with DCMU, lending further support for a biotically-controlled process, or one linked to PSI in the light. A similar response was found in a tropical CCA where surface pH was raised 0.6 units at pH 7.8, while only 0.25 at pH 8.1 from dark to light (Hofmann et al., 2016). Recent studies using boron isotopes have also shown that pHc increases at low (~0.5 to 1.0 pH units at 7.64 pH) compared to ambient pH (~0.2 to 0.8 pH units at 8.08) in coralline algae (Cornwall et al., 2017a). Also using boron isotopic proxies, the rhodolith Neogoniolithon sp. was found to increase pHc 1.26 pH units at pH 7.64, an almost 2-fold greater Δ pH (0.85) than under ambient (8.19) pH (Donald et al., 2017). An increase in pH within the DBL relative to the bulk seawater could be a consequence of greater CO2 diffusion and/or lower export of cellular H+ as the plasma membrane proton motive force reverses under OA (Taylor et al., 2012). Alternatively, the carbonate equilibria shift towards CO2 under lower pH may increase CA ext-facilitated CO2 uptake resulting in more H+ being neutralized by OH- in the DBL, or enhanced cellular acid-base regulation increasing DBL pH.

Biotic control of Δ pH in the DBL without PSI was only consistently observed for two species, Halimeda and Neogoniolithon, although Jania depicted similar, but inconsistent, dynamics to Neogoniolithon. In the presence of DCMU, light/dark DBL Δ pH was only ~0.05 units and in opposite directions. The divergent species-specific patterns of H+ efflux within the DBL with respect to the light can be reconciled by considering their respective morphologies and calcification sites. Halimeda is made up of tightly joined filaments that form a semi-enclosed intercellular space (ICS) interior to appressed swollen end filaments (utricles). At the surface of these appressed cells, small (~3–500 μm; Borowitzka and Larkum, 1987; Peach et al., 2017a) ion diffusion channels form (De Beer and Larkum, 2001) that may have a light-dependent, potentially a proton ATPase pump, independent of PSI that could enhance calcification within the cell wall. Support for a non-PSI light-driven calcification mechanism in this species was provided by greater net calcification rates in the light with DCMU compared to the dark (McNicholl and Koch, unpublished data). Thus, photosynthesis is a major driver for calcification and dissolution processes that have been linked to the formation of distal
cell invaginations increasing their surface area to support $\text{H}^+$ pumping (Pueschel et al., 2005).

Although Udotea, CCA, and Jania did not demonstrate species-specific light/dark $\text{H}^+$ dynamics independent of PSII, they exhibited unique $\text{H}^+$ dynamics relative to the other species. Udotea was the only species that maintained higher thallus surface pH compared to bulk seawater, but no clear increase of pH under OA conditions relative to controls. This may have been due to diffusion limitation of CO$_2$ through filament sheaths, or a greater dependency on HCO$_3^-$ even at low pH. This species has the most enriched C isotope ratio ($\delta^{13}\text{C} = -14\%$) amongst the five species, and lowered $P_{\text{max}}$ by 40% when active (ATPase) ion transport was inhibited (Zweng et al., 2018), perhaps explaining why it was able to raise pH at the DBL in the light, but did not respond to greater DIC availability. CCA had a slower 2-phase increase in pH at the thallus surface in response to light. The shift in pH change with a steeper slope was coincident with a short-term stabilization or decline in surface O$_2$ concentration, followed by a rapid increase in O$_2$ flux. This response may be reflective of an initial active HCO$_3^-$ uptake mechanism, followed by rapid CA$_\text{ext}$-driven DIC uptake. Jania responded similarly to irradiance, with a rapid 2-phase pH change corresponding to O$_2$ flux and an O$_2$ decrease or plateau between phases. While the 2-phase O$_2$/H$^+$ dynamics exhibit a similar pattern at 8.1 and 7.65 pH, the efficiency of pH change to O$_2$ flux was greater at low pH, consistent with a more rapid conversion of HCO$_3^-$ to CO$_2$ by CA$_\text{ext}$ due to a shift in the DIC chemical equilibria.

The combination of similar O$_2$ dynamics at 8.1 and 7.65 pH, despite

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**Fig. 4.** pH light and dark thalli surface dynamics over the 300 s incubation under ambient and low pH for each species (a = Neogoniolithon, b = Jania, c = CCA, d = Halimeda, e = Udotea). The pH response in the light for all species fit an exponential rise to a maximum single 3-parameter model with the exception of CCA that fit a linear model. In the dark, pH decline over time was fit to a 3-parameter hyperbolic decay model with the exception of Halimeda that had an exponential decay function with a linear combination. Models, parameters and fits are presented in Table 4. Each point is the mean of 3 sequential runs (Fig. 2).
a 3-fold increase in [CO$_2$] in the latter, $\delta^{13}$C isotopic ratios of the species examined, rates of DBL pH change upon illumination at the thalli surface, and DIC modeling imply the use of CCMs that simultaneously support photosynthesis and calcification (Fig. 6). The CCMs are most likely comprised of a HCO$_3$– uptake mechanism with a H$^+$ symport and/or OH– antiport, or other Na$^+$/Ca$^{2+}$ transport systems (Gimmler, 2000; Raven and Hurd, 2012; Taylor et al., 2012). CA$_{ext}$ that enhances diffusive uptake of CO$_2$ (20–65%; Zweng et al., 2018), presumably in combination with proton pumps, may also be important (Fig. 6a). Based on our microsensor study, and applying equilibrium assumptions (CO$_2$SYS), under ambient pH (~8.25, 5.8 nM H$^+$) in the light (Fig. 6a), HCO$_3$– and CO$_2$ provide DIC for photosynthesis and all DIC species (~1494:323:6 μmol kg$^{-1}$ HCO$_3$–:CO$_3^{2-}$:CO$_2$) are available for calcification through diffusive and/or paracellular pathways. Photosynthesis also contributes ATP directly and via oxidative phosphorylation of photosynthates for energy-dependent ion transport and organic substrates important for crystal nucleation, lowering activation energy, and serving as catalysts, such as acid-rich proteins (Bilan and Usov, 2001; Borowitzka, 1982; Von Euw et al., 2017). Cellular CO$_2$ dehydrogenated by CA$_{ext}$ is taken up by diffusion and immediately sequestered for photosynthesis or, because of its “leakiness” out of the cell for a CCM (Raven and Beardall, 2016), cellular CO$_2$ is hydrogenated by CA$_{int}$ to HCO$_3$– and H$^+$ (Fig. 6a). As part of this reaction, OH– is available to neutralize H$^+$ in the cell wall or DBL. HCO$_3$– is transported to the chloroplast for photosynthesis (or potentially used for calcification) and H$^+$ is sequestered by the cell or removed to maintain acid-base regulation. HCO$_3$– uptake may also be driven by other symports/antiports (e.g., Na$^+$/Cl$^-$) and H$^+$/OH$^-$ fluxes regulate acid/base conditions within the cells. The morphology of different macroalgal species provide variations on the role of diffusive versus active ion transport options into and out of the calcification site. For example, *Halimeda* species possess diffusive pathways to DIC, but appear to have a H$^+$ pump to expel protons out of the inter-cellular calcification site. Ion transport through cell walls or mucilage sheaths (e.g., *Udotea*) are primarily constrained by diffusion gradients, DBL microchemistry, potentially cell wall constituents, and/or are driven by ion transport across the plasmalemma. One rhodophyta species (*Neogoniolithon*), and another less

### Table 4

<table>
<thead>
<tr>
<th>Sp</th>
<th>pH</th>
<th>Equation</th>
<th>R$^2$</th>
<th>y0</th>
<th>a</th>
<th>b</th>
<th>c</th>
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<td></td>
<td>Light</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neo</td>
<td>A</td>
<td>$y = y_0 + a^<em>(1−\exp(−b</em>x))$</td>
<td>0.95</td>
<td>8.1</td>
<td>0.059</td>
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<tr>
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<td>L</td>
<td>$y = y_0 + a^<em>(1−\exp(−b</em>x))$</td>
<td>0.97</td>
<td>7.64</td>
<td>0.101</td>
<td>0.017</td>
<td>na</td>
</tr>
<tr>
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<td>A</td>
<td>$y = y_0 + a^<em>(1−\exp(−b</em>x))$</td>
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<td>8.06</td>
<td>0.18</td>
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</tr>
<tr>
<td>Jan</td>
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<td>$y = y_0 + a^<em>(1−\exp(−b</em>x))$</td>
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<td>0.29</td>
<td>0.0145</td>
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<td>8.17</td>
<td>0.0002</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CCA</td>
<td>A</td>
<td>$f = y_0 + a^*x$</td>
<td>0.98</td>
<td>7.65</td>
<td>0.0003</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Hal</td>
<td>A</td>
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<td>0.98</td>
<td>8.1</td>
<td>0.314</td>
<td>0.0105</td>
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<tr>
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<td>7.8</td>
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<td>8.04</td>
<td>0.134</td>
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<tr>
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<td>Dark</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neo</td>
<td>A</td>
<td>$y = y_0 + (a*b)/(b + x)$</td>
<td>0.93</td>
<td>8.1</td>
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<td>0.82</td>
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<tr>
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<td>8.03</td>
<td>0.272</td>
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<tr>
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<td>0.95</td>
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<td>7.59</td>
<td>0.179</td>
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<tr>
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<td>A</td>
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<td>0.96</td>
<td>7.9</td>
<td>0.615</td>
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<tr>
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<td>L</td>
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<td>7.54</td>
<td>0.837</td>
<td>0.016</td>
<td>0.0011</td>
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<td>0.96</td>
<td>8.05</td>
<td>0.158</td>
<td>25.06</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Udot L</td>
<td>$y = y_0 + (a*b)/(b + x)$</td>
<td>0.95</td>
<td>7.69</td>
<td>0.148</td>
<td>20.66</td>
<td>na</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Dynamics of light/dark pH cycles run at (a) ambient and (b) low pH in the presence of the photosystem II inhibitor DCMU. All runs were initiated in the light (300 s) followed by dark (300 s) periods. Three sequential replicated light/dark cycles are shown for *Neogoniolithon* and *Halimeda*. a 3-fold increase in [CO$_2$] in the latter, $\delta^{13}$C isotopic ratios of the species examined, rates of DBL pH change upon illumination at the thalli surface, and DIC modeling imply the use of CCMs that simultaneously support photosynthesis and calcification (Fig. 6). The CCMs are most likely comprised of a HCO$_3$– uptake mechanism with a H$^+$ symport and/or OH$^-$ antiport, or other Na$^+$/Ca$^{2+}$ transport systems (Gimmler, 2000; Raven and Hurd, 2012; Taylor et al., 2012). CA$_{ext}$ that enhances diffusive uptake of CO$_2$ (20–65%; Zweng et al., 2018), presumably in combination with proton pumps, may also be important (Fig. 6a). Based on our microsensor study, and applying equilibrium assumptions (CO$_2$SYS), under ambient pH (~8.25, 5.8 nM H$^+$) in the light (Fig. 6a), HCO$_3$– and CO$_2$ provide DIC for photosynthesis and all DIC species (~1494:323:6 μmol kg$^{-1}$ HCO$_3$–:CO$_3^{2-}$:CO$_2$) are available for calcification through diffusive and/or paracellular pathways. Photosynthesis also contributes ATP directly and via oxidative phosphorylation of photosynthates for energy-dependent ion transport and organic substrates important for crystal nucleation, lowering activation energy, and serving as catalysts, such as acid-rich proteins (Bilan and Usov, 2001; Borowitzka, 1982; Von Euw et al., 2017). Cellular CO$_2$ dehydrogenated by CA$_{ext}$ is taken up by diffusion and immediately sequestered for photosynthesis or, because of its “leakiness” out of the cell for a CCM (Raven and Beardall, 2016), cellular CO$_2$ is hydrogenated by CA$_{int}$ to HCO$_3$– and H$^+$ (Fig. 6a). As part of this reaction, OH$^-$ is available to neutralize H$^+$ in the cell wall or DBL. HCO$_3$– is transported to the chloroplast for photosynthesis (or potentially used for calcification) and H$^+$ is sequestered by the cell or removed to maintain acid-base regulation. HCO$_3$– uptake may also be driven by other symports/antiports (e.g., Na$^+$/Cl$^-$) and H$^+$/OH$^-$ fluxes regulate acid/base conditions within the cells. The morphology of different macroalgal species provide variations on the role of diffusive versus active ion transport options into and out of the calcification site. For example, *Halimeda* species possess diffusive pathways to DIC, but appear to have a H$^+$ pump to expel protons out of the inter-cellular calcification site. Ion transport through cell walls or mucilage sheaths (e.g., *Udotea*) are primarily constrained by diffusion gradients, DBL microchemistry, potentially cell wall constituents, and/or are driven by ion transport across the plasmalemma. One rhodophyta species (*Neogoniolithon*), and another less
consistently \((Jania)\), showed evidence that they possess a PSII-independent light triggered proton pump supported by positive net calcification in the dark and in the light with DCMU (McNicholl and Koch, unpublished data).

Based on the present study, under low pH in the light (Fig. 6b), photosynthesis continues to sustain a high pH at the thalli surface even though \([H^+]\) increases on average \(~130\%\) in the DBL (13.3 nM H\(^+\)) when pH increases from 7.65 to 7.91. As the carbonate equilibria shifts \((-1822:192:17 \text{ μmol kg}^{-1} \text{HCO}_3^-:\text{CO}_3^{2-}:\text{CO}_2\) and CO\(_2\) is taken up for photosynthesis, H\(^+\) are neutralized by OH\(^-\) which can raise the DBL pH (Fig. 6b). Higher DBL \([H^+]\) could also constrain cellular H\(^+\) efflux involved in acid-base regulation, or as part of a HCO\(_3^-\) uptake mechanism, due to a lower concentration gradient at the plasmalemma reversing the proton motive force (Fig. 6b). Further, upregulation of a HCO\(_3^-\)/OH\(^-\) antiport cannot be ruled out (Fig. 6b). Therefore, CO\(_2\) uptake by diffusion, H\(^+\) sequestration through HCO\(_3^-\) dehydration and CO\(_2\) uptake, and/or OH\(^-\) efflux to the DBL are all photosynthesis-driven mechanisms that could raise the DBL pH and \(\Omega_{\text{CaCO}_3}\) at macroalgal calcification sites under OA in the light (Fig. 6b). These results support observations of sustained calcification in the light by these species under OA even though equilibria chemistry (CO2SYS) predicts a 47% decrease in DBL \([\text{CO}_3^{2-}]\) based on averages in this study.

In the dark, H\(^+\) are not efficiently removed from the DBL and calcifying space even at ambient pH (Fig. 6c). Compounding this potential acidification problem is an internal accumulation of respiratory CO\(_2\) that would need to diffuse out of the cell, or be hydrolyzed to HCO\(_3^-\) and H\(^+\). Subsequently, the H\(^+\) would need to be exported across the plasma membrane for acid-base regulation, and the respiratory CO\(_2\) sequestered in CaCO\(_3\). In addition to acid-base issues within and external to the cell, energetics from photosynthesis, and potentially organics for nucleation and other processes required to sustain optimal conditions for calcification are reduced. Although we measured modest increases in pH at the thalli surface relative to bulk seawater in the dark, only two rhodophyte species (Neogoniolithon, Jania) were able to maintain net positive calcification in the dark at ambient pH (McNicholl and Koch, unpublished data). Under OA in the dark, all species examined herein (McNicholl and Koch, unpublished data) and by Kamenos et al. (2013) (Lithothamnion glaciale) exhibited net dissolution at average pH of 7.72 (19.4 nM H\(^+\)) when CO\(_3^{2-}\) levels declined \((-1984:128:26 \text{ μmol kg}^{-1} \text{HCO}_3^-:\text{CO}_3^{2-}:\text{CO}_2\)).

Thus, for marine calcifying macroalgae, photosynthesis is critical to remove H\(^+\) from the DBL, promote ion flux that supports CCMs and DBL chemistry, and cellular acid-base homeostasis, particularly under OA conditions. While some rhodophytes sustain H\(^+\) pumps and calcification in the dark, at low pH in the dark, ion pumps likely become overwhelmed by high DBL CO\(_2\) and H\(^+\), even though they maintain the DBL pH above the bulk seawater. Certainly, additional studies are required to further identify tropical macroalgal species-specific
mechanisms of DIC uptake for photosynthesis and how these are linked to ion transport mechanisms that impact calcification. Also, there is great interest in how increases in seawater [H+] and DIC speciation with ongoing OA will influence these mechanisms on short and long time scales that incorporate acclimation and adaptation.

Declaration of Competing Interest
None.

Acknowledgements
We recognize the following graduate and undergraduate students (Regina Zweng, Chris Johnson, Dr. Kate Peach, Kim McFarlane) for field assistance and staff at Unisense for microsensor training and troubleshooting. Two anonymous reviews significantly improved this manuscript. Discussions with Drs. John Raven, Kim Yates, Mary Bison and George Bowes assisted in our interpretation of the data and proposed mechanisms; Dr. Raven is especially recognized for kindly reviewing the manuscript and providing constructive comments based on his expansive knowledge of macroalgal physiology. This research was funded by the National Science Foundation, United States through Grant #140-381.

Appendix A. Supplementary data
Supplementary data to this article can be found at https://doi.org/10.1016/j.jembe.2019.151208.

References
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