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Mechanisms of bicarbonate use influence the photosynthetic carbon dioxide sensitivity of tropical seagrasses

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Abstract

The photosynthetic bicarbonate (HCO_3^-) use properties of three widely distributed tropical seagrasses were compared using a series of laboratory experiments. Photosynthetic rates of *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* were monitored in an enclosed chamber while being subjected to shifts in pH and dissolved inorganic carbon. Specific mechanisms of seagrass HCO_3^- use were compared by examining the photosynthetic effects of the carbonic anhydrase inhibitor acetazolamide (AZ). All seagrasses increased photosynthetic rates with reduced pH, suggesting a large effect of dissolved aqueous carbon dioxide ($CO_{2(aq)}$). However, there was considerable interspecific variation in pH response. *T. testudinum* was highly sensitive, increasing photosynthetic rates by 100% as the pH was reduced from 8.2 to 7.4, whereas rates in *H. wrightii* and *S. filiforme* increased by only 20% over a similar range, and displayed prominent photosynthetic plateaus, indicating an increased capacity for HCO_3^- use. Additional incubations that manipulated [HCO_3^-] under constant [$CO_{2(aq)}$] support these findings, as only *H. wrightii* and *S. filiforme* increased photosynthetic rates with increasing [HCO_3^-]. *T. testudinum* responded to AZ addition, indicating that carbonic anhydrase enzymes facilitate limited HCO_3^- use. *H. wrightii* and *S. filiforme* showed no response to AZ, suggesting alternate, more efficient mechanisms of HCO_3^- use. Estimated kinetic parameters, $K_s(CO_2)$ and V_{max}, revealed interspecific variation and further support these conclusions. Variation in photosynthetic pH responses and AZ sensitivity indicate distinctions in the carbon use properties of seagrasses exposed to similar environmental conditions. These results suggest that not all seagrasses will similarly respond to future increases in $CO_{2(aq)}$ availability. Attention towards potential shifts in competitive interactions within multispecific seagrass beds is warranted.

The growth and survival of submerged vegetation depends upon the ability to acquire the resources necessary to support photosynthetic carbon fixation. While light levels are commonly invoked as the primary resource that regulates photosynthesis, the supply rate of dissolved inorganic carbon (DIC) has also been demonstrated as an important factor (Beer and Koch 1996; Zimmerman et al. 1997). Photosynthetic carbon limitation has been observed in a wide variety of marine plants (seagrasses), many of which can double photosynthetic production with increases in dissolved aqueous carbon dioxide ($CO_{2(aq)}$) (Durako 1993; Zimmerman et al. 1997; Invers et al. 2001). These findings are fundamental towards understanding the factors that regulate seagrass productivity, and furthermore, have implications for the future functioning of these systems in regards to climate change, as it is suggested that most seagrasses will benefit from anticipated increases in oceanic DIC concentrations (Beer and Koch 1996; Invers et al. 2002; Hall-Spencer et al. 2008).

Photosynthetic carbon limitation in the marine environment results from a number of physicochemical factors that restrict the supply rate of inorganic carbon to the leaf surfaces of seagrasses. In addition to limited diffusion rates (Stumm and Morgan 1981) and the presence of unstirred boundary layers at the leaf surface (Koch 1994), the primary inorganic carbon source for photosynthesis ($CO_{2(aq)}$) is in limited supply in seawater, comprising only 1% (roughly 10–15 μmol L^{-1}) of the DIC pool. At normal pH, the bicarbonate ion (HCO_3^-) is the most abundant inorganic carbon species, accounting for nearly 90% of the DIC pool, whereas the remaining 9% is represented by the carbonate ion (CO_3^{2-}). Thus, despite an overall abundance of DIC in seawater (2.2 mmol L^{-1}), the carbonate species most essential for seagrass photosynthesis is in least supply.

Seagrasses have adapted to low seawater [$CO_{2(aq)}$] by employing a variety of mechanisms to use the more abundant HCO_3^- ion to meet photosynthetic carbon demand. Bicarbonate use centers around the operation of at least one (or more) of the following mechanisms: (1) extracellular dehydration of HCO_3^- into $CO_{2(aq)}$ via membrane-bound carbonic anhydrase (CA) enzymes (James and Larkum 1996; Beer and Rehnberg 1997; Bjork et al. 1997); or (2) electrogenic proton (H^+) extrusion into an unstirred boundary layer adjacent to the leaf surface, which facilitates CA activity or HCO_3^-/H^+ cotransport (Hellblom et al. 2001; Uku et al. 2005). Photosynthetic responses to DIC manipulations have revealed some degree of HCO_3^- use in the seagrasses *Thalassia testudinum*, *Zostera marina*, *Posidonia oceanica*, *Cymodocea nodosa*, and *Phyllospadix torreyi* (Durako 1993; Beer and Rehnberg 1997; Invers et al. 2001). Bicarbonate use has been further identified for *Posidonia australis*, *Cymodocea serrulata*, *Halophila ovalis*, *Halodule wrightii*, *Cymodocea rotundata*, *Thalassia hemprichii*, *Thalassondendron cilatum*, *Syringodium isoetifolium*, and *Enhalus acoroides* (James and

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Larkum 1996; Schwarz et al. 2000; Uku et al. 2005). However, while widely documented, prior research highlights interspecific variation in the extent of HCO_3^- use among seagrasses (Schwarz et al. 2000; Invers et al. 2001; Uku et al. 2005). For example, in a comparative study, Invers et al. (2001) suggest an increased capacity of HCO_3^- use in Mediterranean as compared to Pacific seagrasses, as evidenced by differential photosynthetic responses to increasing [DIC]. This suggests that the effects of future increases in $CO_{2(aq)}$ availability on seagrass performance might be species specific, and depend upon mechanisms of HCO_3^- use.

Variation in HCO_3^- use can have implications towards how disparate groups of submerged vegetation respond to changes in $[CO_{2(aq)}]$. For example, while seagrasses use HCO_3^- to meet photosynthetic demand, research has shown that they employ relatively inefficient acquisition mechanisms compared to macroalgae (Durako 1993; Beer and Koch 1996; Invers et al. 1999). Thus, photosynthetic rates are carbon saturated for many macroalgae and carbon limited for many seagrasses. In the context of globally increasing $[CO_{2(aq)}]$, seagrass responses may outweigh macroalgal responses, potentially shifting the competitive balance between these photosynthetic groups (Beer and Koch 1996). With interspecific variation in seagrass HCO_3^- use (Bjork et al. 1997; Uku et al. 2005) and varying photosynthetic responses to increased $CO_{2(aq)}$ (Schwarz et al. 2000; Invers et al. 2001), certain seagrasses may benefit more from increasing $[CO_{2(aq)}]$ relative to others, similarly influencing competitive interactions among sympatric species.

Evidence of variation in seagrass HCO_3^- use may be further revealed through interspecific divergence in stable carbon isotope values ($\delta^{13}C$) (Raven et al. 1995; Hemminga and Mateo 1996; Raven et al. 2002). As HCO_3^- is isotopically distinct from dissolved $CO_{2(aq)}$ (0‰ and −9‰, respectively), seagrasses with different degrees of HCO_3^- use might display varying isotopic signatures under similar environmental conditions. For example, Campbell and Fourqurean (2009) document consistent interspecific variation in the $\delta^{13}C$ value of three sympatric seagrasses across South Florida, suggesting different mechanisms of HCO_3^- use. Links between HCO_3^- acquisition and photosynthetic $CO_{2(aq)}$ sensitivity have yet to be established for these widely distributed seagrasses that commonly form mixed-species meadows. Comparing the $CO_{2(aq)}$ sensitivity of co-occurring seagrasses from similar environments will provide an increasingly detailed view of which species will respond the most to future increases in $CO_{2(aq)}$ supply.

This study directly compares HCO_3^- use in three tropical seagrasses and tests the hypothesis that differential photosynthetic responses to increases in $[CO_{2(aq)}]$ are driven by variation in carbon acquisition properties. Photosynthetic responses to changes in $[CO_{2(aq)}]$ and $[HCO_3^-]$ concentrations are tested using a series of closed-cell DIC manipulations. The CA inhibitor acetazolamide (AZ) is used to detail specific mechanisms of HCO_3^- use. We provide evidence to suggest that the photosynthetic benefits of globally increasing $[CO_{2(aq)}]$ may be greater for certain species relative to others.

Methods

Bicarbonate use was compared for the most common seagrasses in the tropical western Atlantic Ocean: *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme*. Samples of all three species were collected from a single seagrass meadow near Stock Island (1 m depth) in Key West, Florida (24.55°N, 81.75°W). During May 2011, plant fragments consisting of two or more vertical shoots (along with the connected horizontal rhizomes) were carefully excavated and transported back to the laboratory in aerated, dark coolers filled with ambient seawater. Seagrasses were stored under dim light (20–30 μmol quanta m^{-2} s^{-1}) until required for laboratory experiments that were conducted within 72 h of collection. All leaf material remained healthy, green, and lesion free over this period. Furthermore, under natural seawater conditions, we did not detect any significant increase in seagrass respiration over the storage period.

Laboratory experiments—Bicarbonate use was assessed by monitoring photosynthetic rates (O_2 evolution) of each seagrass species at varying pH and DIC concentrations, following the methods of Durako (1993). Seagrass leaf segments of all species were exposed to one of two distinct seawater manipulations: pH variation at constant [DIC] (*Series I*), or $[HCO_3^-]$ variation at constant $[CO_{2(aq)}]$ (*Series II*). Each seawater series uniquely manipulated DIC, and comparisons of photosynthetic rates at each incubation treatment revealed patterns of HCO_3^- use. Within the *Series I* incubations, variation in pH altered the relative proportions of the carbonate species $CO_{2(aq)}$, HCO_3^-, and CO_3^{2-} (Table 1). As photosynthetic rates are monitored across varying pH, plateaus in this response curve indicate some degree of HCO_3^- use (Beer et al. 1977, 1980*a*; Invers et al. 2001).To conclusively provide further evidence of HCO_3^- use, additional seawater incubations (*Series II*) were conducted that held $[CO_{2(aq)}]$ constant and manipulated $[HCO_3^-]$; thus, observed increases in photosynthetic rates with $[HCO_3^-]$ support bicarbonate use (Durako 1993). To assess specific mechanisms of HCO_3^- use, the inhibitor AZ was used in conjunction with the seawater manipulations to evaluate the presence of enzymes that can dehydrate HCO_3^- into $CO_{2(aq)}$. AZ is a membrane-impermeable inhibitor that has been previously used to assess extracellular CA activity in a wide variety of marine macrophytes (Beer and Rehnberg 1997; Bjork et al. 1997; Uku et al. 2005). Both *Series I* and *Series II* incubations were conducted with and without AZ.

Seawater incubations were replicated ($n = 4$) with leaf segments from separate seagrass shoots (not connected via a horizontal rhizome). Four separate shoots of each seagrass species were selected, and two leaf segments (approximately 3 mg dry weight [dry wt]) were excised with a razor from the middle, epiphyte-free portion of the rank 2 leaf (second youngest). One of the leaf segments was subjected to the *Series I* incubations, whereas the other was subjected to the *Series II* incubations. All incubations were then repeated with the CA inhibitor. Thus, each leaf segment was exposed to an AZ and a non-AZ treatment of

Table 1. Measured and calculated carbonate parameters of the *Series I* and *II* incubation media. [$CO_{2(aq)}$] and [HCO_3^-] were calculated from the Excel macro CO_2SYS (Lewis and Wallace 1998) utilizing the dissociation constants of Dickson and Millero (1987). An Orion 4-star pH meter calibrated with NBS standards (relative accuracy ± 0.002) was utilized for all pH measurements.

Series I

pH	CO_2 (mmol L^{-1})	HCO_3^- (mmol L^{-1})
7.2	0.125	2.050
7.4	0.080	2.079
7.6	0.050	2.084
7.8	0.032	2.065
8.0	0.020	2.021
8.2	0.012	1.945
8.4	0.007	1.830
8.6	0.004	1.670

Series II

pH	DIC	CO_2 (mmol L^{-1})	HCO_3^- (mmol L^{-1})
7.8	0.762	0.011	0.715
8.2	2.381	0.012	2.083
8.6	6.571	0.012	4.962

either *Series I* or *Series II* incubation media. Due to differences in leaf morphology, 3 mg leaf segments consisted of a single piece of *T. testudinum*, whereas leaf segments from *H. wrightii* and *S. filiforme* consisted of three smaller 1 mg segments. All leaf segments were placed in synthetic, unbuffered seawater (Instant Ocean, salinity 35‰) for 12 h prior to experimentation to allow for wound repair.

Incubation media—*Series I* incubations consisted of eight seawater treatments in which the pH was adjusted from 7.2 to 8.6 (in units of 0.2), whereas the total DIC concentration was held constant (2.2 mmol L^{-1}). Such a range encompasses the natural variation in pH experienced by these plants at the site of collection (8.0–8.4, J. E. Campbell pers. obs.), and includes a number of reduced pH values replicating anticipated $CO_{2(aq)}$ forecasts extending to the year 2300 (Caldeira and Wickett 2003). Personal observations at the collection site have revealed that pH naturally varies over both a diurnal and seasonal cycle (Campbell and Fourqurean 2011). On average, daily pH ranged from a minimum of 8.02 in the morning to a maximum of 8.24 in the late afternoon. Average seasonal variation was over a similar range. Synthetic seawater (Instant Ocean, salinity 35‰) was titrated in 500 mL glass incubation bottles to various pH values (± 0.02 National Bureau of Standards [NBS] scale) by adding either 2 mol L^{-1} HCl or carbonate-free NaOH. Once the target pH was reached, each bottle was quickly sealed with a glass stopper and placed in a temperature-controlled water bath (25°C). *Series I* incubations primarily altered $CO_{2(aq)}$ concentrations, and to a lesser extent HCO_3^- concentrations (Table 1). However, note that CO_3^{2-} concentrations also changed, resulting in a 30% shift in total alkalinity over the pH range (7.2–8.6). Due to the inability of seagrasses to directly utilize CO_3^{2-} for photosynthesis (Raven 1970; Prins and Elzenga 1989; Maberly 1992), shifts in photosynthetic rates resulting from the different *Series I* incubations are primarily due to changing $CO_{2(aq)}$ concentrations, and to a lesser extent changing HCO_3^- concentrations. The *Series II* incubations were then conducted to specifically look at photosynthetic responses to bicarbonate. *Series II* incubations consisted of three seawater treatments in which both pH and total DIC were varied to produce changes in [HCO_3^-] while holding [$CO_{2(aq)}$] constant (near air-saturated equilibrium values) (Durako 1993). Synthetic seawater was acidified to a pH of 4 with 2 mol L^{-1} HCl and vigorously stirred and bubbled with 100% N_2 gas for 4 h to remove all DIC. Individual DIC concentrations ranging from ~ 0.75 mmol L^{-1} to 6.57 mmol L^{-1} were achieved by adding measured amounts of $NaHCO_3$, and the final pH was adjusted to pre-calculated values with carbonate-free NaOH. The incubation media for this series resulted in increasing [HCO_3^-], which ranged from ~ 0.7 mmol L^{-1} to 5.0 mmol L^{-1}, while [$CO_{2(aq)}$] was relatively constant, ranging from 10 μmol L^{-1} to 12 μmol L^{-1} (*see* Table 1). A stock solution of the CA inhibitor was prepared by dissolving 0.4445 g of AZ in 100 mL of 25 mmol L^{-1} NaOH, yielding a final AZ concentration of 20 mmol L^{-1}.

Photosynthetic measurements—Seagrass photosynthetic rates under various pH and DIC treatments were determined by O_2 evolution under stirred, temperature-controlled conditions. The order of the various seawater treatments was randomized for each incubation series. Photosynthetic measurements were conducted within a 2.5 mL reaction chamber (Hansatech model DW1) connected to a calibrated, Clark-type polargraphic oxygen sensor (Hansatech model S1). Irradiance was provided by dual 75 W halogen bulbs that illuminated the reaction chamber from both sides, providing 500 μmol quanta m^{-2} s^{-1} photosynthetically active radiation (as measured with a small, calibrated planar light sensor oriented perpendicular to the light field). The temperature was maintained at 25°C by a refrigerated recirculating water bath. Stir rates were identical for all species, and further testing revealed that they were vigorous enough to saturate photosynthetic rates with respect to water motion. Prior to administering the incubation series, respiration rates for each leaf segment were measured by monitoring O_2 consumption within the reaction chamber under darkened conditions with synthetic seawater (pH 8.2). Incubation media (2 mL) was then serially injected into the reaction chamber, and oxygen production was carefully monitored under illumination for 6 min. Oxygen measurements within the chamber were recorded every minute during the incubation period, and immediately plotted to ensure linear rates of O_2 production. After the 6 min incubation period, the chamber was opened and the seawater was carefully removed with a syringe (leaving the seagrass leaf material within the chamber). The chamber was flushed once with the next randomly selected incubation media, and then slowly refilled and resealed for the next incubation. All incubations for each seagrass species (both *Series I* and *II*) were then repeated in the presence of the CA inhibitor by

adding 10 μL of the 20 mmol L^{-1} AZ stock solution to the 2 mL of incubation media in the reaction chamber. This yielded a final AZ concentration of 100 μmol L^{-1}, a concentration previously used to inhibit CA activity under similar experimental conditions (Bjork et al. 1997). CA activity was evidenced by significant reductions in plant photosynthetic rates after AZ addition. All leaf segments were measured (length and width) and dried for 48 h at 80°C to determine dry weight. Photosynthetic measurements were calculated from the linear portions of the [O_2] vs. time curves, and were corrected for variation in leaf respiration. Rates of gross photosynthesis are reported as mg O_2 g^{-1} dry wt h^{-1}. Kinetic parameters were calculated for all species using the *Series I* data set with $CO_{2(aq)}$ as the primary enzymatic substrate. The half-saturation constant (K_s) and maximum photosynthetic rate (V_{max}) were estimated using a linear transformation (Hanes method) of the Michaelis–Menten equation (Dowd and Riggs 1965).

Statistical procedures—For all statistical procedures, gross photosynthetic rates of individual leaf segments at a given seawater treatment represented a unit of observation. A one-way repeated-measures analysis of variance (ANOVA) was used to test for photosynthetic responses to pH (*Series I*) and HCO_3^- concentrations (*Series II*) within each species and inhibitor treatment. When assumptions of normality failed, a repeated-measures ANOVA was conducted on ranked values. The effect of the inhibitor AZ on photosynthetic rates was tested with a paired *t*-test within each level of pH or [HCO_3^-] for each species. When assumptions of normality failed, a Wilcoxon signed-rank test was used. Significant declines in photosynthetic rates within each seawater treatment indicated the presence of extracellular, membrane-bound CA activity. For interspecific comparisons, a two-way repeated-measures ANOVA (within the AZ-free, control incubations) was used to assess species-specific variation in photosynthetic pH responses (pH × species interactions). Interspecific distinctions in kinetic parameters (K_s and V_{max}) were evaluated with a one-way ANOVA. The effects of AZ on kinetic parameters within each species were evaluated with a paired *t*-test.

Results

Series I*: pH variation at constant [DIC]*—For all seagrass species, photosynthetic rates significantly increased at low pH (Fig. 1; repeated-measures ANOVA, $p < 0.001$ for all species). For *T. testudinum*, photosynthetic rates were higher (~ 2×) at a pH of 7.4 compared to normal seawater pH of 8.2. *S. filiforme* and *H. wrightii* similarly increased photosynthetic rates over the same pH range, however to a lesser extent (~ 1.2×). Increasing photosynthetic rates do correspond to increasing $CO_{2(aq)}$ availability (Fig. 2); however, note a weak relationship at low [$CO_{2(aq)}$] for *H. wrightii* and *S. filiforme*. AZ addition significantly reduced photosynthetic rates in *T. testudinum*, however at only three of the eight pH values (7.4, 7.6, and 8.2). For these three incubation media, *T. testudinum* photosynthetic rates were reduced by 33%, 22%, and 23%, respectively. Furthermore, a trend of decreasing CA activity

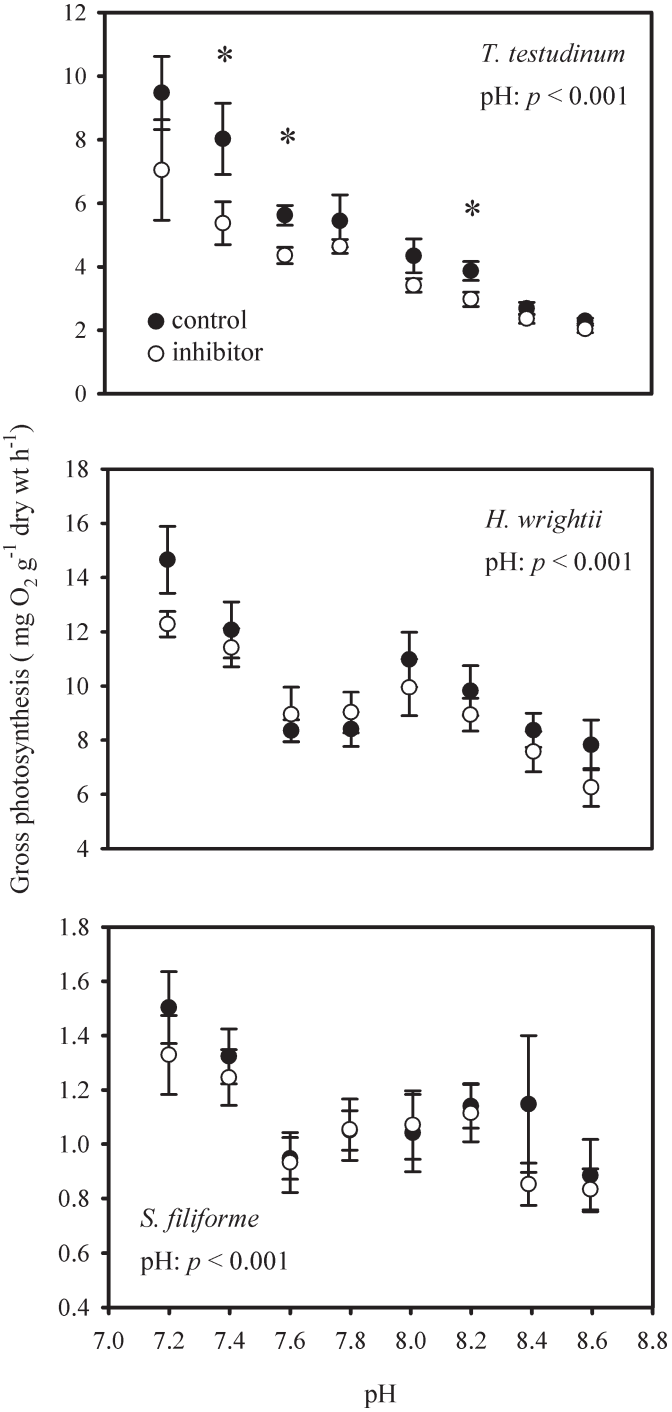

Fig. 1. *Series I* photosynthetic pH response curves for *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme*. Closed and open symbols represent gross photosynthetic rates (means ± 1 SE; $n = 4$) for the control and inhibitor (AZ) incubations, respectively. Significant differences in photosynthetic rates with AZ addition are indicated with an asterisk ($p < 0.05$). Displayed *p*-values indicate the results of a one-way repeated-measures ANOVA within the control incubations (closed symbols).

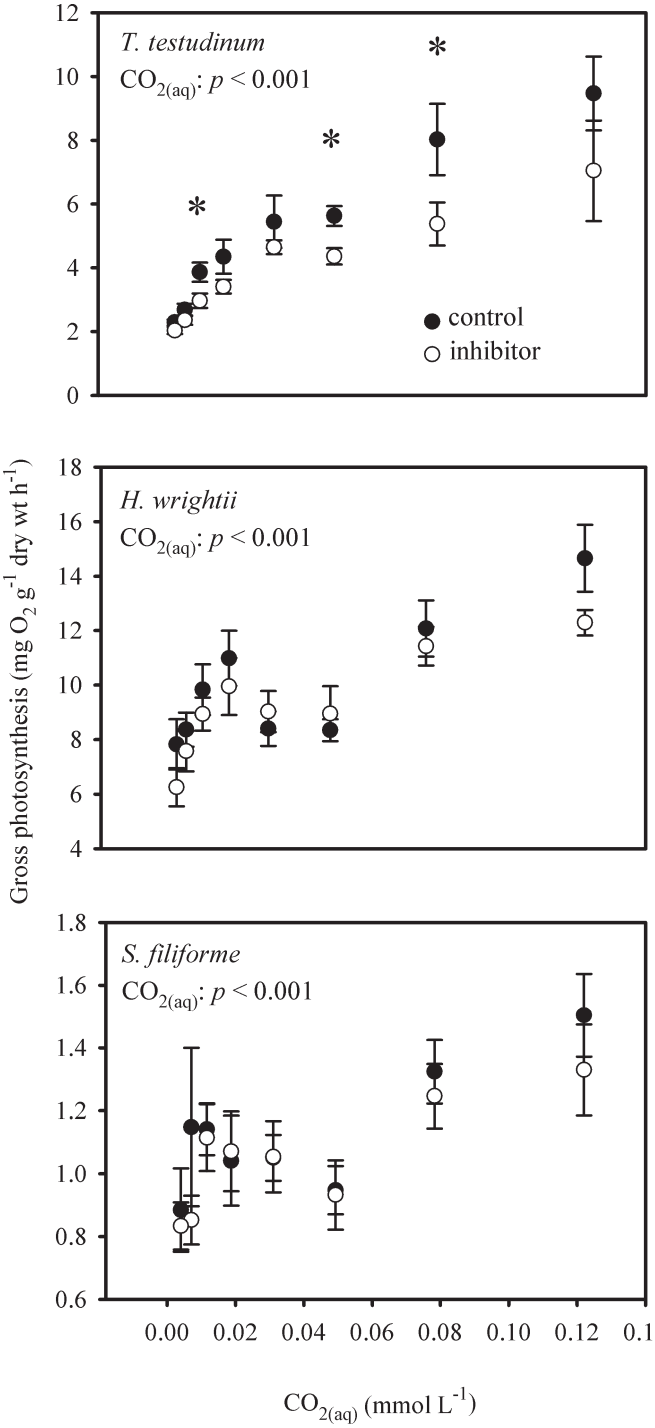

Fig. 2. *Series I* photosynthetic $CO_{2(aq)}$ response curves for *T. testudinum*, *H. wrightii*, and *S. filiforme*. Closed and open symbols represent photosynthetic rates (means ± 1 SE; $n = 4$) for the control and inhibitor (AZ) incubations, respectively. Significant differences in photosynthetic rates with AZ addition are indicated with an asterisk ($p < 0.05$). Displayed *p*-values indicate the results of a one-way repeated-measures ANOVA within the control incubations (closed symbols).

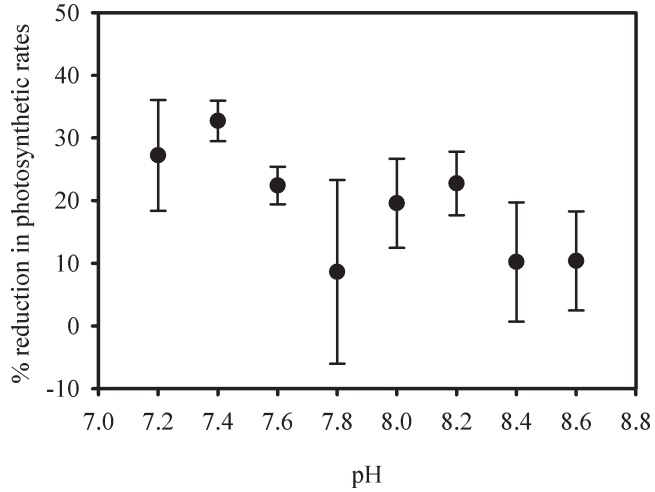

Fig. 3. *Series I* relative photosynthetic importance of carbonic anhydrase (CA) for *T. testudinum*. CA importance is expressed as percent reduction in gross photosynthetic rates (means ± 1 SE; $n = 4$) after inhibitor addition at each respective pH.

with increasing pH was observed (Fig. 3). AZ had no effect on the photosynthetic rates of *S. filiforme* or *H. wrightii* at any pH value.

Interspecific comparisons in photosynthetic response curves reveal a significant pH × species interaction, with *T. testudinum* displaying increased responsiveness to pH relative to *H. wrightii* and *S. filiforme* (Fig. 4). Photosynthetic rates of *T. testudinum* moderately plateau in the pH range from 7.6 to 8.2, while *H. wrightii* and *S. filiforme* both display prominent plateaus from pH 7.6 to 8.4. Across the *T. testudinum* plateau, $[CO_{2(aq)}]$ varies by 76%, whereas photosynthetic rates vary by 38%. Across the *H. wrightii* and *S. filiforme* plateaus, $[CO_{2(aq)}]$ varies by 86%, whereas photosynthetic rates remain constant. Thus, whereas photosynthetic rates tended to scale linearly with pH for *T. testudinum*, both *H. wrightii* and *S. filiforme* displayed a curvilinear relationship. Further note that mass-specific photosynthetic rates of *S. filiforme* were lower than those for *T. testudinum* and *H. wrightii*, potentially attributable to the cylindrical leaf morphology of this species.

Estimated half-saturation constants (K_s) were significantly higher for *T. testudinum* as compared to *H. wrightii* and *S. filiforme* (ANOVA, $p < 0.001$) (Table 2). Estimated V_{max} was significantly distinct among all species, with *H. wrightii* displaying the highest values and *S. filiforme* displaying the lowest values. Within each species, the addition of AZ reduced the calculated V_{max} of *T. testudinum* (*t*-test, $t = 5.015$, df = 3, $p = 0.015$). AZ did not significantly alter the K_s value for *T. testudinum*, nor did it alter any of the kinetic parameters for *H. wrightii* or *S. filiforme*.

Series II*: $[HCO_3^-]$ variation at constant $[CO_{2(aq)}]$*—Increases in $[HCO_3^-]$ under constant $[CO_{2(aq)}]$ had no effect on the photosynthetic rates of *T. testudinum* (Fig. 5). In contrast, both *H. wrightii* and *S. filiforme* significantly increased photosynthetic rates in response to increases in

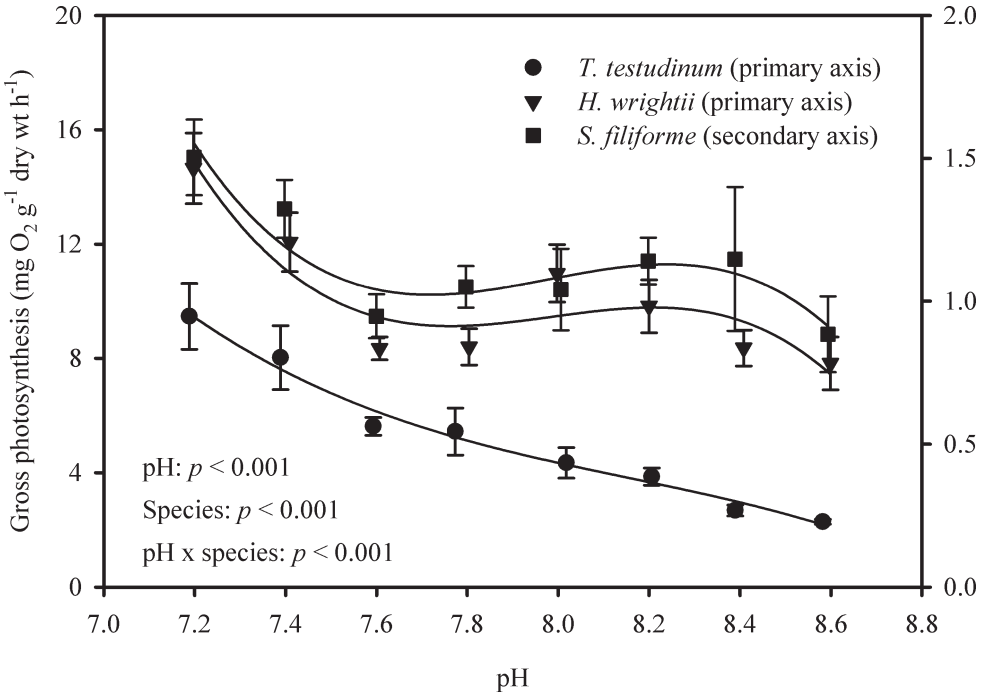

Fig. 4. *Series I* photosynthetic pH response curves for *T. testudinum*, *H. wrightii*, and *S. filiforme* for the control incubations. Closed symbols represent gross photosynthetic rates (means ± 1 SE; $n = 4$). Data for *S. filiforme* are plotted on a secondary axis. Displayed *p*-values indicate the results of a two-way repeated-measures ANOVA. Lines represent curvilinear fits to photosynthetic data for each species (*T. testudinum*, $r^2 = 0.98$; *H. wrightii*, $r^2 = 0.88$; *S. filiforme*, $r^2 = 0.84$).

$[HCO_3^-]$ (repeated-measures ANOVA, $p < 0.05$). Post hoc analysis revealed that only the lowest and highest $[HCO_3^-]$ were significantly different. With the addition of AZ, photosynthetic rates of *H. wrightii* remained significantly responsive to $[HCO_3^-]$; however, the response of *S. filiforme* was insignificant after AZ addition. Photosynthetic HCO_3^- responses in *T. testudinum* remained unaltered after AZ addition, with no significant increase in photosynthetic rates with $[HCO_3^-]$.

Discussion

This study demonstrates interspecific variation in the HCO_3^- use properties of three tropical seagrasses. All species increased photosynthetic rates with reductions in pH; however, the magnitude and shape of these photosynthetic responses varied between species. Thus, it is shown that while HCO_3^- use occurs, the mechanisms of acquisition are species specific, supporting prior findings (Invers et al. 2001) and suggesting that not all seagrasses similarly respond to increases in $CO_{2(aq)}$ supply.

Reductions in pH tend to increase the photosynthetic rates of marine macrophytes due to increases in the availability of $CO_{2(aq)}$. These responses have been documented for a wide variety of submerged plants (Sand-Jensen and Gordon 1984; Invers et al. 1997, 1999), and reflect a relatively inefficient use of HCO_3^- as compared to $CO_{2(aq)}$. Within the *Series I* incubations, shifting the pH from 7.2 to 8.6 increases the ratio of HCO_3^- to $CO_{2(aq)}$, primarily due to exponential declines in $[CO_{2(aq)}]$. Thus, large variation in plant photosynthetic rates over this range can be attributed to shifts in $CO_{2(aq)}$ availability, whereas minor variation (a photosynthetic plateau) may indicate substantial HCO_3^- use.

Table 2. Calculated photosynthetic kinetic parameters from linear transformations of the Michaelis–Menten equation. The Hanes transformation $[(C_s/v) = (K_s/V_{max}) + (1/V_{max})C_S]$ was used (Dowd and Riggs 1965), where Cs = the substrate $CO_{2(aq)}$ concentration (from *Series I*) and v = the photosynthetic rate (from *Series I*). Within a species, bold parameter values indicate a significant effect ($p < 0.05$, paired *t*-test) of AZ addition. Among species, the results of post hoc tests for interspecific comparisons of a given kinetic parameter are indicated. Different letters indicate significant differences ($p < 0.05$, Holm–Sidak).

Species	K_s	K_s (AZ)	*p*-value	V_{max}	V_{max} (AZ)	*p*-value
T. testudinum	0.025[a]	0.021[a]	0.702	**10.892[a]**	**7.555[a]**	**0.015**
H. wrightii	0.012[b]	0.009[b]	0.311	14.453[b]	12.576[b]	0.235
S. filiforme	0.009[b]	0.006[b]	0.14	1.499[c]	1.335[c]	0.208

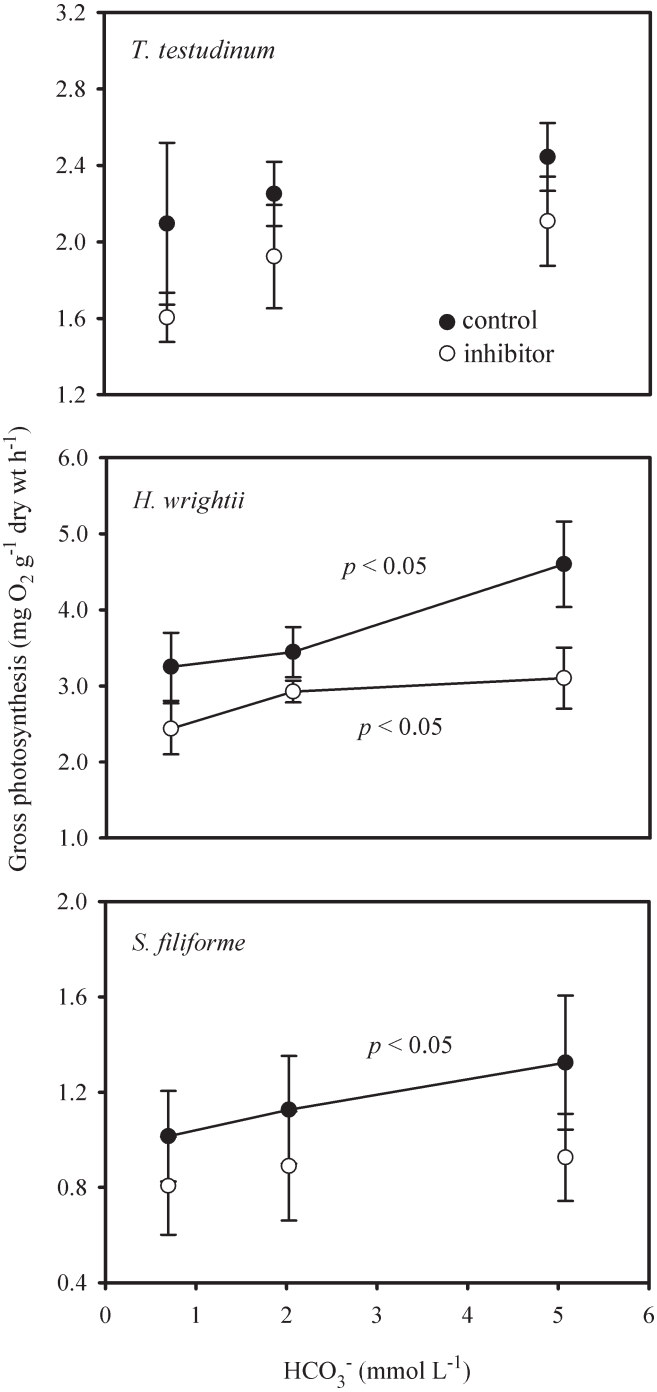

Fig. 5. *Series II* photosynthetic HCO_3^- response curves for *T. testudinum*, *H. wrightii*, and *S. filiforme*. Closed and open symbols represent photosynthetic rates (means ± 1 SE; $n = 4$) for the control and inhibitor incubations, respectively. Solid lines designate significant trends in photosynthetic responses to increasing HCO_3^- concentrations ($p < 0.05$, repeated-measures ANOVA).

Plateaus in the photosynthetic pH response curve have been used to infer HCO_3^- use in marine macrophytes (Beer et al. 1980*b*; Sand-Jensen and Gordon 1984; Durako 1993). Such plateaus are evident in our data set, as across the entire pH range in the *Series I* incubations, declines in photosynthetic rates did not scale to exponential declines in $[CO_{2(aq)}]$, suggesting HCO_3^- use in all three species. However, note that relative to *T. testudinum*, *H. wrightii*, and *S. filiforme* display prominent plateaus, with photosynthetic rates that are insensitive to $[CO_{2(aq)}]$ at high pH (Fig. 2). These trends demonstrate that *H. wrightii* and *S. filiforme* display an increased capacity to use HCO_3^-, resulting in decreased photosynthetic pH sensitivity. These conclusions are supported by the results of the *Series II* incubations, in which $[HCO_3^-]$ was increased by 6-fold and $[CO_{2(aq)}]$ were held constant near present-day, air-equilibrated values. While *T. testudinum* displayed no increase in photosynthetic rates, both *H. wrightii* and *S. filiforme* positively responded as $[HCO_3^-]$ increased from 0.7 mmol L^{-1} to 5.0 mmol L^{-1}, supporting relatively inefficient HCO_3^- use in *T. testudinum* (Durako 1993). The basis for these distinctions might result from differential expression of the various HCO_3^- use mechanisms (Invers et al. 2001).

Calculated half-saturation constants show good agreement with prior estimates, and suggest that photosynthetic rates of *T. testudinum* are currently carbon limited with respect to $CO_{2(aq)}$ under natural conditions. Durako (1993) estimated the $K_S(CO_2)$ values of *T. testudinum* under similar experimental conditions, and demonstrated a relatively high affinity for $CO_{2(aq)}$ ($K_S \approx 3$–18 μmol L^{-1}). Our calculated $K_S(CO_2)$ values ranged from 16 μmol L^{-1} to 31 μmol L^{-1}, and further demonstrate a high affinity for $CO_{2(aq)}$ as compared to other submerged vegetation, which typically display $K_S(CO_2)$ values ranging from 70 to 300 μmol L^{-1} (Sand-Jensen and Gordon 1984). Thus, in the context of increasing $CO_{2(aq)}$ concentrations, calculated K_s constants indicate photosynthetic benefits for *T. testudinum*. Both *H. wrightii* and *S. filiforme* displayed significantly lower $K_S(CO_2)$ values as compared to *T. testudinum* (12 and 9 μmol L^{-1}, respectively), indicating that photosynthetic rates were near carbon saturation with respect to $CO_{2(aq)}$ under natural conditions (typically ranging from 10–15 μmol L^{-1}). The low $K_S(CO_2)$ values of *H. wrightii* and *S. filiforme* may further account for the observation that photosynthetic rates only increased by 20% over the 7.4–8.2 pH range as compared to the 100% increase over the same range for *T. testudinum*.

Treatment with AZ significantly reduced the calculated V_{max} parameter for *T. testudinum*, suggesting that photosynthetic rates in *T. testudinum* are partially dependent upon extracellularly bound CA enzymes, which facilitate HCO_3^- dehydration into $CO_{2(aq)}$. In comparison, the calculated V_{max} parameter for *H. wrightii* and *S. filiforme* was not altered by AZ, and suggest that extracellular CA enzymes do not play a role in HCO_3^- use in these species; however, note that AZ sensitivity in *H. wrightii* has been previously documented (Uku et al. 2005). Photosynthetic rates of *T. testudinum* significantly expressed AZ sensitivity at three pH values whereby photosynthetic rates declined by 26% on average. This reduction is relatively moderate as compared to reductions found in other AZ-sensitive species, which range up to nearly 50–60% for some species (Beer and Rehnberg 1997; Bjork et al. 1997). Furthermore, we find that CA enzymes and HCO_3^- use play a significant

role in supporting photosynthetic rates even at lower pH values (7.4), where photosynthetic rates for *T. testudinum* are strongly regulated by $CO_{2(aq)}$ (Durako 1993). At high pH, CA activity declines (Fig. 3), findings which have been similarly reported in other studies (Invers et al. 1999). CA enzymes are relatively inefficient under alkaline conditions because they can only restore [$CO_{2(aq)}$] to equilibrium values as set by pH (Beer et al. 2002). Thus, at high pH, the equilibrium values of $CO_{2(aq)}$ are too low to support diffusional transport across the plasmalemma, as $CO_{2(aq)}$ is rapidly rehydrated to HCO_3^-. Throughout our *Series I* incubations for *T. testudinum*, we found no evidence of CA activity at pH values above 8.4. This decline in activity, along with declines in $CO_{2(aq)}$, towards high pH is likely responsible for the continued reduction in *T. testudinum* photosynthetic rates above 8.4. Thus, we find limited evidence of HCO_3^- use mechanisms other than membrane-bound CA activity in *T. testudinum*. Conversely, *H. wrightii* (in both *Series I* and *Series II*) and *S. filiforme* (*Series I* only) show no evidence of external CA activity, and demonstrate the ability to maintain photosynthetic rates across a broad pH range (pH 7.6–8.6). In the absence of CA activity, these findings suggest the operation of an alternate HCO_3^- acquisition mechanism (i.e., acidification of leaf boundary layers and H^+/HCO_3^- cotransport). Photosynthetic rates of *H. wrightii* display a high sensitivity to buffered solutions, suggesting that H^+ extrusion and the formation of acidic zones adjacent to the leaf surface facilitate DIC assimilation (Uku et al. 2005). We suggest that a similar mechanism may operate for *S. filiforme* in our study.

Interspecific variation in the HCO_3^- use properties of marine macrophytes might result in ecological distinctions among sympatric species. For temperate seagrasses, species inhabiting high-pH environments with low rates of water exchange demonstrate an increased capacity for HCO_3^- utilization (Invers et al. 2001). Furthermore, Uku et al. (2005) suggest that species inhabiting the high-light environments of the upper intertidal tend to also display efficient mechanisms of bicarbonate acquisition, primarily centered on H^+ extrusion and leaf boundary acidification. The expression of these efficient DIC use mechanisms may confer an advantage upon these species when faced with high midday irradiances (Uku et al. 2005). The interspecific variation presented in this study may also result from ecological distinctions, and reflect species-specific differences in photosynthetic potential and overall growth rates. *H. wrightii* and *S. filiforme* are relatively fast growing seagrass species compared to *T. testudinum*, thus potentially requiring efficient mechanisms of photosynthetic DIC uptake to support the fast growth rates.

Several studies have suggested that seagrasses may display plastic mechanisms of bicarbonate use (Sand-Jensen and Gordon 1984; Invers et al. 2001), which may be advantageous for populations that experience large shifts in DIC availability. Early work with macroalgae from the Swedish coast has demonstrated significant plasticity in HCO_3^- use in response to increasing pH (Larsson et al. 1990; Axelsson et al. 1995), suggesting that similar capabilities might exist for seagrasses. While disparities between our findings and those of Uku et al. (2005) in regards to the AZ sensitivity of *H. wrightii* suggest plastic responses, there is currently a lack of empirical data from manipulative experiments that explicitly examines plasticity in seagrass carbon acquisition. We submit that additional research is currently needed to further assess the ability of seagrasses to up- or down-regulate various bicarbonate use mechanisms in response to shifting DIC concentrations. In light of global changes in oceanic DIC availability, such work would be especially informative to help assess the future responses of submerged macrophytes.

Oceanic CO_2 concentrations are forecasted to increase nearly 3-fold over the next century (Brewer 1997). Such rapid changes in the chemical composition of the marine environment have been unprecedented over the past 300 million years (Honisch et al. 2012), and have the potential to alter the productivity and functioning of marine vegetation. However, here we demonstrate that within the group of seagrasses (and even for co-occurring species experiencing similar environmental conditions), the extent of HCO_3^- use varies and the photosynthetic CO_2 response of marine vegetation is not uniform. In the context of tropical Atlantic seagrasses, a future with increased atmospheric pCO_2 and decreased oceanic pH may lead to greater benefits for *T. testudinum* compared to *H. wrightii* and *S. filiforme*. Prior research has examined the role that shifts in DIC availability might play for competitive interactions between seagrasses and macroalgae (Beer and Koch 1996; Invers et al. 1999; Mvungi et al. 2012). Here, we highlight the role that seagrass physiology plays in potentially regulating climate change responses, urging additional study in regards to the ecological implications of these distinctions. One potential outcome is that the large, non-photosynthetic belowground biomass of *T. testudinum* imposes high respiratory demands, and increases whole-plant light requirements relative to other smaller-bodied species such as *H. wrightii* and *S. filiforme*. Thus, under similar environmental conditions, increased $CO_{2(aq)}$ may improve the carbon balance of *T. testudinum*, elevating its competitive ability with respect to smaller-bodied species.

As research directed towards studying the effects of climate change on marine macrophytes grows, we suggest an increased awareness of how physiological HCO_3^- use properties regulate the photosynthetic responses of specific species, and consideration of how these differential responses might influence competitive interactions in multispecific communities.

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