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The Effects of Carbon Dioxide Fertilization on the Ecology of Tropical Seagrass Communities

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THE EFFECTS OF CARBON DIOXIDE FERTILIZATION ON THE ECOLOGY OF TROPICAL SEAGRASS COMMUNITIES

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by

Justin E. Campbell

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To: Dean Kenneth Furton
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This dissertation, written by Justin E. Campbell, and entitled The effects of carbon dioxide fertilization on the ecology of tropical seagrass communities, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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ABSTRACT OF THE DISSERTATION

THE EFFECTS OF CARBON DIOXIDE FERTILIZATION ON THE ECOLOGY OF TROPICAL SEAGRASS COMMUNITIES

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Increasing atmospheric CO_2 concentrations associated with climate change will likely influence a wide variety of ecosystems. Terrestrial research has examined the effects of increasing CO_2 concentrations on the functionality of plant systems; with studies ranging in scale from the short-term responses of individual leaves, to long-term ecological responses of complete forests. While terrestrial plants have received much attention, studies on the responses of marine plants (seagrasses) to increased $CO_{2(aq)}$ concentrations remain relatively sparse, with most research limited to small-scale, *ex situ* experimentation. Furthermore, few studies have attempted to address similarities between terrestrial and seagrass responses to increases in $CO_{2(aq)}$. The goals of this dissertation are to expand the scope of marine climate change research, and examine how the tropical seagrass, *Thalassia testudinum* responds to increasing $CO_{2(aq)}$ concentrations over multiple spatial and temporal scales.

Manipulative laboratory and field experimentation reveal that, similar to terrestrial plants, seagrasses strongly respond to increases in $CO_{2(aq)}$ concentrations. Using a novel field technique, *in situ* field manipulations show that over short time

scales, seagrasses respond to elevated $CO_{2(aq)}$ by increasing leaf photosynthetic rates and the production of soluble carbohydrates. Declines in leaf nutrient (nitrogen and phosphorus) content were additionally detected, paralleling responses from terrestrial systems. Over long time scales, seagrasses increase total above- and belowground biomass with elevated $CO_{2(aq)}$, suggesting that, similar to terrestrial research, pervasive increases in atmospheric and oceanic $CO_{2(aq)}$ concentrations stand to influence the productivity and functionality of these systems. Furthermore, field experiments reveal that seagrass epiphytes, which comprise an important component of seagrass ecosystems, additionally respond to increased $CO_{2(aq)}$ with strong declines in calcified taxa and increases in fleshy taxa.

Together, this work demonstrates that increasing $CO_{2(aq)}$ concentrations will alter the functionality of seagrass ecosystems by increasing plant productivity and shifting the composition of the epiphyte community. These results have implications for future rates of carbon storage and sediment production within these widely distributed systems.

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INTRODUCTION

There has been increasing concern over the response of the Earth's biosphere to changes in the global climate over the past 35 years. Since the Industrial Revolution, anthropogenic activities such as fossil fuel combustion and deforestation have resulted in a 25% increase in atmospheric carbon dioxide concentrations (Houghton and Woodwell 1989). The direct and indirect effects of these carbon emissions on natural ecosystems have been widely documented, and much attention has been directed towards the influence of rising CO_2 levels on plant growth and development. Within terrestrial systems, elevated CO_2 concentrations can influence a variety of plants via alterations in a number of physiologically-based performance parameters, such as increases in photosynthetic, water-use, and light-use efficiency (Bazzaz 1990; Drake et al. 1997). These small-scale physiological responses have the potential to influence larger-scale mechanisms, altering both community and ecosystem level processes (Bazzaz 1990). Our understanding of how terrestrial systems respond to additional CO_2 has benefitted from advances in research methodology which allows for the experimental manipulation of CO_2 concentrations across expanded spatial scales and under realistic environmental conditions. Alterations in primary production, community structure, and nutrient cycling have all received considerable attention (Melillo et al. 1993; Delucia et al. 1999; Smith et al. 2000; Norby et al. 2005; Luo et al. 2006), and suggest that increasing atmospheric CO_2 concentrations will likely influence plant systems across multiple scales.

In contrast, the effects of CO_2 enrichment on marine plants (seagrasses) has received relatively limited attention. The oceans represent a substantial long-term sink for atmospheric CO_2 (Archer et al. 1998; Sabine et al. 2004), and have thus far absorbed nearly 30% of anthropogenic carbon emissions (Feely et al. 2004). As such, dissolved

CO_2 ($CO_{2(aq)}$) concentrations are forecast to nearly triple in the oceanic surface waters by the year 2100 (Brewer 1997). Similar to terrestrial plants, submerged vegetation inhabiting shallow coastal areas will be exposed to increases in $CO_{2(aq)}$ with continued climate change. The large-scale implications of altered $CO_{2(aq)}$ concentrations on the ecology of seagrass systems have yet to be thoroughly explored.

Research on the responses of seagrasses to increased $CO_{2(aq)}$ concentrations has demonstrated physiological responses similar to terrestrial vegetation. Increases in photosynthetic rates under elevated $CO_{2(aq)}$ concentrations have been documented for a large number of seagrasses (Durako 1993; Beer and Koch 1996; Invers et al. 1997; Zimmerman et al. 1997; Invers et al. 1999; Invers et al. 2001; Enriquez and Rodriguez-Roman 2006). Mesocosm work has shown increases in soluble carbohydrates and overall improvements towards plant carbon balance with $CO_{2(aq)}$ enrichment (Zimmerman et al. 1997; Invers et al. 2002; Palacios and Zimmerman 2007). These trends suggest that, similar to terrestrial vegetation, seagrasses may potentially respond to increasing $CO_{2(aq)}$ concentrations over multiple scales.

Seagrass meadows play an important role in organic carbon production and nutrient cycling in many coastal regions around the world (Orth *et al.* 2006). The importance of seagrass meadows as sites of carbon sequestration is now being realized, as they account for nearly 15% of all oceanic production, and roughly 50% of carbon burial (Duarte and Chiscano 1999). Seagrass ecosystem carbon (C) storage rivals the storage of C in terrestrial forests and mangrove ecosystems on an areal basis, as most of the C storage is in the sediment organic matter (Fourqurean et al. 2012). Furthermore, the export of fixed carbon from seagrass ecosystems substantially contributes to the carbon

budget of adjacent ecosystems (Heck *et al.* 2008). Thus, understanding the ecological implications of $CO_{2(aq)}$ related changes in the functionality (particularly as it relates to biomass production) of these coastal systems remains an important topic.

Seagrass $CO_{2(aq)}$ research is largely restricted to *ex situ* mesocosm conditions, which tend to focus on physiological responses over limited scales. Thus, relative to terrestrial research, we have a poor understanding of how small-scale physiological responses of CO_2 enrichment translate into larger-scale responses. *Ex situ* research is confined in its applicability to natural systems due to space limitations and the presence of 'chamber effects', as suggested for many terrestrial experiments (Arp 1991; Ainsworth and Long 2005). Shifts in biomass production, carbon storage, and nutrient cycling have been unexplored, arising from difficulties in experimentally manipulating $CO_{2(aq)}$ levels in the field. Techniques for *in situ* $CO_{2(aq)}$ manipulations in the marine environment are currently needed to fully evaluate conclusions derived from prior mesocosm experimentation. The goals of this dissertation are to develop and implement new techniques of submerged *in situ* $CO_{2(aq)}$ manipulation, and use this method to examine how seagrasses response to additional $CO_{2(aq)}$ supply across multiple spatial and temporal scales. Furthermore, this dissertation evaluates the conclusions from prior seagrass $CO_{2(aq)}$ research, and examines how prior findings are represented over longer time scales in natural communities.

Chapter I presents the results of a series of short term laboratory experiments which detail the photosynthetic carbon acquisition properties of three tropical seagrasses in South Florida. Using a series of pH and DIC manipulations, Chapter I documents short term photosynthetic responses to $CO_{2(aq)}$ enrichment, and further details varying, species-

specific mechanisms of seagrass carbon use by observing photosynthetic responses to a series of enzyme inhibitor treatments. Given these results, Chapter II continues to document evidence of varying carbon use strategies by examining natural variation in the isotopic composition of the same three seagrass species in South Florida. Using long-term monitoring data from the South Florida region, Chapter II documents consistent interspecific variation in the stable carbon isotope values of these seagrasses over large spatial scales, providing further evidence that the interspecific variation in carbon acquisition seen from the laboratory manipulations may be reflected in the $\delta^{13}C$ values of seagrasses across South Florida.

Chapter III details the technique of submerged $CO_{2(aq)}$ enrichment that will be used to explore the long-term effects of seagrass carbon enrichment, and Chapter IV describes the initial responses of a shallow seagrass bed to 6 months of continuous enrichment. Overall, $CO_{2(aq)}$ enrichment had no immediate effect on seagrass growth, however strong declines in the leaf nutrient content were detected. Furthermore, we find that after 6 months, $CO_{2(aq)}$ enriched seagrasses had elevated concentrations of belowground soluble carbohydrates. These documented trends in Chapter IV suggest a potential link between nutrient availability and the ability of seagrasses to respond to additional $CO_{2(aq)}$ supply; thus, nutrient-limited conditions may have constrained seagrass growth responses, and instead promoted increased carbohydrate storage. The link between seagrass nutrient availability and carbohydrate storage was further investigated in Chapter V, which uses prior monitoring data to examine the relationship between seagrass nutrient content and belowground carbohydrate content within several regions across Florida. Overall, these field surveys suggest that low nutrient availability might

increase seagrass carbohydrate storage, potentially explaining documented trends in Chapter IV.

Chapter VI continues with this theme, and examines whether nutrient availability influences the responses of *T. testudinum* to $CO_{2(aq)}$ enrichment. Both nutrient and carbon availability were manipulated in a fully factorial design for a full year at the same field location of Chapter IV. Contrary to previous conclusions, nutrient supply had no influence on seagrass responsiveness to *in situ* $CO_{2(aq)}$ enrichment. Furthermore, the longer duration of this experiment did reveal slight increases in seagrass biomass under carbon enrichment, suggesting that the responses documented in the shorter experiment of Chapter IV are distinct from longer-term responses documented in Chapter V.

Lastly, Chapter VII examines the effects of $CO_{2(aq)}$ enrichment on seagrass epiphytes, an important component of seagrass systems. Using the same carbon/nutrient manipulation experiment of the previous chapter, detailed observations of the epiphyte community reveal dramatic declines in calcified taxa with $CO_{2(aq)}$ addition. Associated with these declines are increases in the abundance of fleshy, uncalcified epiphytes with carbon enrichment. Overall, these observations suggest that seagrass epiphytes might be highly responsive to ocean acidification, with particular sensitivity exhibited by calcified groups.

The seven chapters of this dissertation represent a comprehensive documentation of the responses of a tropical seagrass community to *in situ* CO_2 enrichment. Responses are detailed across a wide range of temporal and spatial scales, and generally support the conclusions from prior mesocosm research. Furthermore, this work documents a number of responses found in terrestrial plant CO_2 research, suggesting that similar CO_2 response

mechanisms may operate for submerged plant communities. This work demonstrates that the effects of small-scale photosynthetic responses are manifested at larger scales within natural seagrass beds, which tend to display long-term increases in overall aboveground biomass.

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CHAPTER I

SEAGRASSES DISPLAY INTERSPECIFIC VARIATION IN PHOTOSYNTHETIC CARBON UPTAKE PROPERTIES

ABSTRACT

The photosynthetic bicarbonate (HCO_3^-) use properties of three widely-distributed, sympatric tropical seagrasses were compared using a series of laboratory experiments. Photosynthetic rates of *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* were monitored in a closed reaction chamber while being subjected to manipulations of both pH and dissolved inorganic carbon (DIC). Specific mechanisms of seagrass HCO_3^- use (carbonic anhydrase activity) were compared by examining the effect of a specific inhibitor, acetazomide (AZ) on photosynthetic rates. Under constant DIC concentrations, all three species increased photosynthetic rates in response to reduced pH, suggesting a large effect of dissolved $CO_{2\,(aq)}$ on seagrass photosynthetic rates, particularly towards low pH. However, considerable interspecific variation was present in photosynthetic pH responses. *Thalassia testudinum* was highly sensitive, increasing photosynthetic rates by 100% as the pH was reduced from 8.2 - 7.4. In comparison, photosynthetic rates in *H. wrightii* and *S. filiforme* increased by only 20% over a similar range, and displayed prominent photosynthetic plateaus in the pH response curve, indicating an increased capacity to use HCO_3^- relative to *T. testudinum*. Additional incubations that manipulated [HCO_3^-] under constant [$CO_{2(aq)}$] support these findings; as only *H. wrightii* and *S. filiforme* significantly increased photosynthetic rates with increasing [HCO_3^-]. Observed mechanisms of HCO_3^- use were additionally species-specific, and corresponded to the degree of HCO_3^- use. The photosynthetic rates of *T. testudinum* responded to AZ addition, indicating the use of carbonic anhydrase enzymes; while *H. wrightii* and *S. filiforme* showed no response to AZ, suggesting alternate

mechanisms of HCO_3^- use. Interspecific variation in photosynthetic pH response curves and AZ sensitivity indicate marked differences 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, thus attention towards potential shifts in competitive interactions within multispecific seagrass beds is warranted.

INTRODUCTION

The growth and survival of submerged vegetation depends upon their ability to acquire the resources necessary to support photosynthetic carbon fixation. While light levels are commonly invoked as the primary resource which 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 carbon dioxide, $CO_{2(aq)}$ (Durako 1993; Beer and Koch 1996; Invers et al. 1997; Zimmerman et al. 1997). 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; Zimmerman et al. 1997; Invers et al. 2001; Invers et al. 2002; Hall-Spencer et al. 2008; Jiang et al. 2010).

Photosynthetic carbon limitation in the marine environment results from a number of physiochemical 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 μM) of the DIC pool. At normal pH, the bicarbonate ion (HCO_3^-) is the most abundant carbon source, accounting for nearly 90% of the DIC pool, while the remaining 9% is represented by the carbonate ion (CO_3^{2-}). Thus, despite an overall abundance of DIC in seawater (2.2 mM), 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; Invers et al. 1999); or 2) electrogenic proton (H^+) extrusion into an unstirred boundary layer adjacent to the leaf surface, which facilitates CA activity or HCO_3^- / H^+ co-transport (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* (Sandjensen and Gordon 1984; 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 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 (Bjork et al. 1997; 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; evidenced by differential photosynthetic responses to increasing [DIC]. These findings suggest 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 inefficient acquisition mechanisms relative 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, and 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). Given prior research which demonstrates interspecific variation in the HCO_3^- use of seagrasses (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 globally increasing $[CO_{2(aq)}]$ relative to others, similarly influencing competitive interactions among sympatric species.

Evidence of variation in seagrass HCO_3^- use may also be revealed through interspecific divergence in stable carbon isotope values ($\delta^{13}C$) (Raven et al. 1995; Hemminga and Mateo 1996; Raven et al. 2002b). 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. Campbell & Fourqurean (2009) document consistent interspecific variation in the $\delta^{13}C$ value of 3 sympatric seagrasses across South Florida; suggesting altered 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 which commonly form mixed species meadows. Comparing the $CO_{2(aq)}$ sensitivity of co-occurring seagrasses under similar environmental conditions provides 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 3 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, acetazomide (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 properties were 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 (1m depth) in Key West, Florida, USA (24.55 N, 81.75W). During May 2011, plant fragments consisting of 2 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. All laboratory experiments were conducted within 72 hrs of collection.

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; 1) pH variation at constant [DIC] (*Series I*), or 2) [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; Beer et al. 1980a; Invers et al. 2001). To conclusively provide further evidence of HCO_3^- use, additional seawater incubations (*Series II*) were conducted which 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 specific inhibitor, acetazomide (AZ) was used in conjunction with the seawater manipulations to evaluate the presence of enzymes which 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 (James and Larkum 1996; Beer and Rehnberg 1997; Bjork et al. 1997; Invers et al. 1999; 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 horizontal rhizome). Four separate shoots of each seagrass species were selected, and two leaf segments (approximately 3mg DW) 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, while the other was subjected to the *Series II* incubations. All incubations were then repeated with the carbonic anhydrase inhibitor. Thus, each leaf segment was exposed to an AZ and non-AZ treatment of either *Series I* or *Series II* incubation media. Due to differences in leaf morphology, 3mg leaf segments consisted of a single piece of *T. testudinum*, while leaf segments from *H. wrightii* and *S. filiforme* consisted of 3 smaller 1 mg segments. All leaf segments were placed in synthetic, unbuffered seawater (Instant Ocean, salinity 35 ‰) for 12 hours prior to experimentation to allow for wound repair.

Incubation media

Series I incubations consisted of 8 seawater treatments in which the pH was adjusted from 7.2 - 8.6 (in units of 0.2) while the total DIC concentration was held constant (2.2 mM). Such a range encompasses, 1) the natural variation in pH experienced by these plants at the site of collection (8.0-8.4, personal observation), and 2) includes a number of reduced pH values replicating anticipated $CO_{2(aq)}$ forecasts extending to the year 2300 (Caldeira and Wickett 2003). Synthetic seawater (Instant Ocean, salinity 35‰) was titrated in 500ml glass incubation bottles to various pH values (±0.02 NBS scale) by adding either 2N 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^{o}C). *Series II* incubations consisted of 3 seawater treatment 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 2N HCL and vigorously stirred and bubbled with 100% N_2 gas for 4 hours to remove all DIC. Individual DIC concentrations ranging from ca. 0.75mM - 6.57 mM 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 ca. 0.7mM - 5mM, while $CO_{2(aq)}$ concentrations were relatively constant, ranging from 10*u*M - 12*u*M (see Table 1). A stock solution of the CA inhibitor was prepared by dissolving .4445 g of AZ in 100ml of 25mM NaOH, yielding a final AZ concentration of 20mM.

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 75W halogen bulbs which illuminated the reaction chamber from both sides, providing 500 *u*mol photons m^{-2} s^{-1} PAR. The temperature was maintained at 25°C by a refrigerated recirculating water bath. 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 (2ml) was then serially injected into the reaction chamber, and oxygen production was carefully monitored under illumination for 6 minutes. Oxygen measurements within the chamber were recorded every min 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. The next randomly selected incubation media was then slowly injected into the chamber, which was then resealed for the next incubation period. All incubations for each seagrass species (both *Series I* and *II*) were then repeated in the presence of AZ by adding 10*u*l of the 20mM stock solution to the 2 ml of incubation media injected into the reaction chamber. This yielded a final AZ concentration of 100*u*M, which has been previously utilized to inhibit CA activity under similar experimental conditions (Bjork et al. 1997). Carbonic anhydrase activity was

evidenced by significant reductions in plant photosynthetic rates after AZ addition. All leaf segments were measured (length and width) and dried for 48hrs at 80°C to determine dry weight. All photosynthetic measurements were calculated from the linear portions of the [O_2] versus time curves, and were corrected for variation in respiration. Rates of gross photosynthesis are reported as umol O_2 min^{-1} $mgdw^{-1}$.

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 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 impact 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. Thus, significant declines in photosynthetic rates within each seawater treatment indicated CA activity. For interspecific comparisons, a two-way, repeated-measures ANOVA (within the AZ-free incubations) was used to assess species-specific variation in photosynthetic pH responses (pH x species interactions).

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 (ca. 2x) at a pH of 7.4 compared to normal seawater pH of 8.2. *Syringodium filiforme* and *H. wrightii* similarly increased photosynthetic rates over the same pH range, however to a lesser extent (ca.1.2x). 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 3 of the 8 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 with increasing pH was additionally 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 x 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 - 8.2, while *H. wrightii* and *S. filiforme* both display prominent plateaus from pH 7.6 - 8.4. Across the *T. testudinum* plateau, [$CO_{2(aq)}$] varies by 76% while photosynthetic rates vary by 38%. Across the *H. wrightii* and *S. filiforme* plateaus, [$CO_{2(aq)}$] varies by 86%, while photosynthetic rates remain constant. Thus, while 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 altered cylindrical leaf morphology of this species.

Series II: [HCO_3^-] variation at constant [$CO_{2(aq)}$]

Increases in [HCO_3^-] under constant [$CO_{2(aq)}$] had no impact 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 [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 suggest 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 (Sandjensen and Gordon 1984; Janzen 1986; Durako 1993; Invers et al. 1997; Invers et al. 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 -

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 (photosynthetic plateau) may indicate substantial HCO_3^- use.

Plateaus in the photosynthetic pH response curve have been used to infer HCO_3^- use in marine macrophytes (Beer et al. 1980b; Sandjensen and Gordon 1984; Durako 1993). Such plateaus are evident in our dataset, 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 increasingly 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 utilize 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*m*M - 5.0*m*M, supporting relatively inefficient HCO_3^- use in *T. testudinu*m (Durako 1993). The basis for these distinctions might result from differential expression of the various HCO_3^- use mechanisms, as previously suggested (Invers et al. 2001).

Results from AZ addition suggest that photosynthetic rates in *T. testudinum* are partially dependent upon extracellularly-bound, carbonic anhydrase enzymes, which facilitate HCO_3^- dehydration into $CO_{2(aq)}$. In comparison, photosynthetic rates in *H.*

wrightii and *S. filiforme* were not impacted by AZ, and suggest that extracellular CA enzymes do not play a role in HCO_3^- use in these species; however, note 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). Carbonic anhydrase 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^- co-transport). Photosynthetic rates in *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 from 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. *Halodule wrightii* and *S. filiforme* are relatively fast growing seagrass species compared to *T. testudinum*, thus requiring efficient mechanisms of photosynthetic DIC uptake to support the fast growth rates.

Since the industrial revolution, 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 not all marine vegetation responds similarly. 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*. The ecological implications of these distinctions will certainly require additional study, but we suggest potential shifts in competitive interactions. 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*. Under similar environmental conditions, increased $CO_{2(aq)}$ may improve the carbon balance of *T. testudinu*m, increasing competitive ability.

As research directed towards studying the impact of climate change on marine macrophytes grows, we suggest 1) an increased awareness towards how physiological HCO_3^- use properties regulate the photosynthetic responses of individual species, and 2) consideration of how these differential responses might influence competitive interactions in multispecific communities.

ACKNOWLEDGMENTS

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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.

FIGURE CAPTIONS

Figure 1. *Series I* photosynthetic pH response curves for *T. testudinum*, *H. wrightii*, and *S. filiforme*. Closed and open symbols represent gross photosynthetic rates (means ± 1 SE; n = 4) for the control (AZ-free) 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 measured ANOVA within the AZ-free, control incubations (closed symbols).

Figure 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 (AZ-free) 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 AZ-free, control incubations (closed symbols).

Figure 3. *Series I* carbonic anhydrase (CA) activity for *T. testudinum*. CA activity is expressed as percent reduction in photosynthetic rates (means ± 1 SE; n = 4) after AZ addition at each respective pH.

Figure 4. *Series I* photosynthetic pH response curves for *T.testudinum*, *H. wrightii*, and *S. filiforme* for the AZ-free, 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).

Figure 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 (AZ-free) and inhibitor (AZ) incubations respectively. Solid lines designate significant trends in photosynthetic responses to increasing HCO_3^- concentrations ($p < 0.05$, repeated-measures ANOVA).

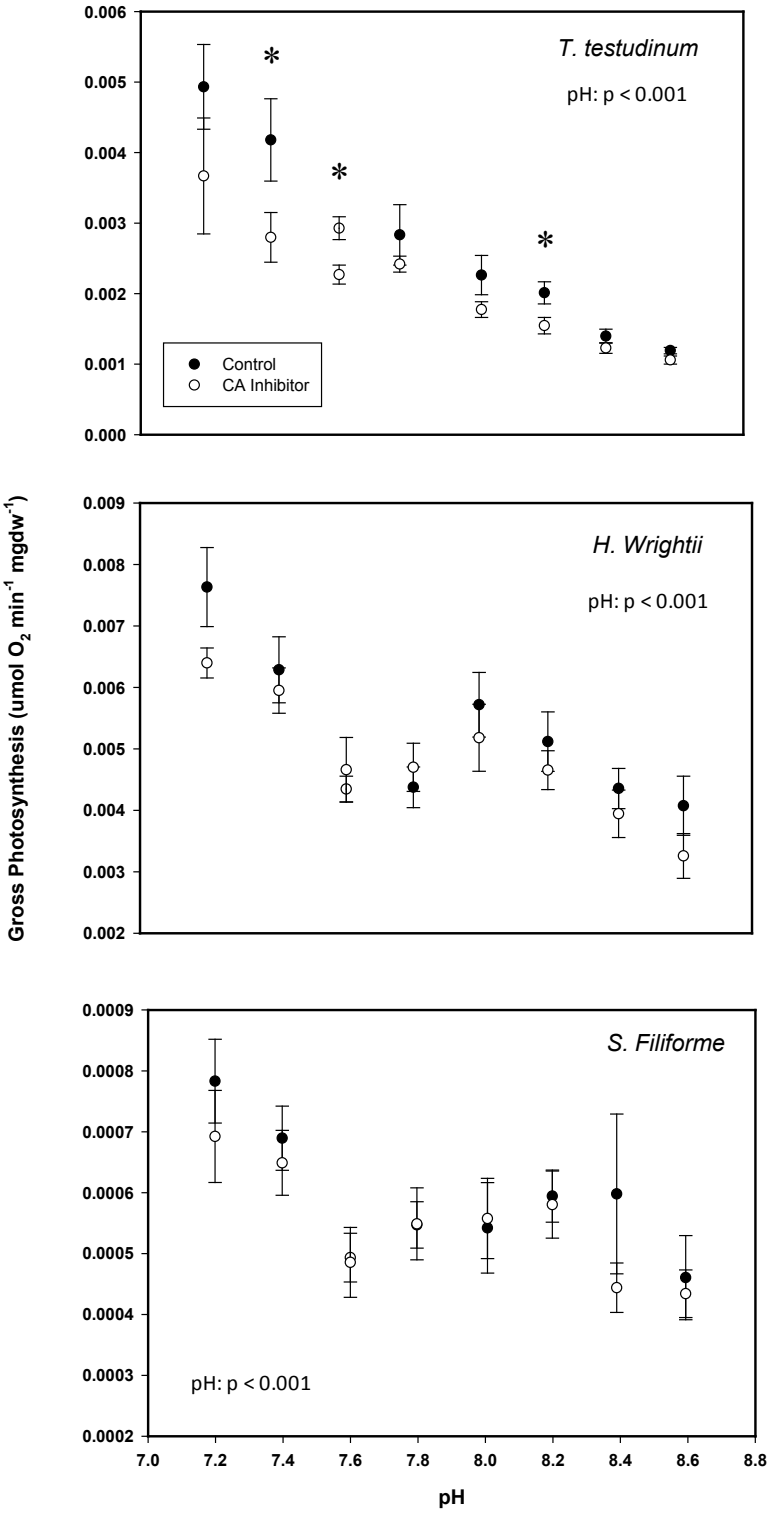
T. testudinum
pH: p < 0.001
Control
CA Inhibitor
H. Wrightii
pH: p < 0.001
S. Filiforme
pH: p < 0.001
Gross Photosynthesis (umol O2 min-1 mgdw-1)
pH

FIGURE 1.

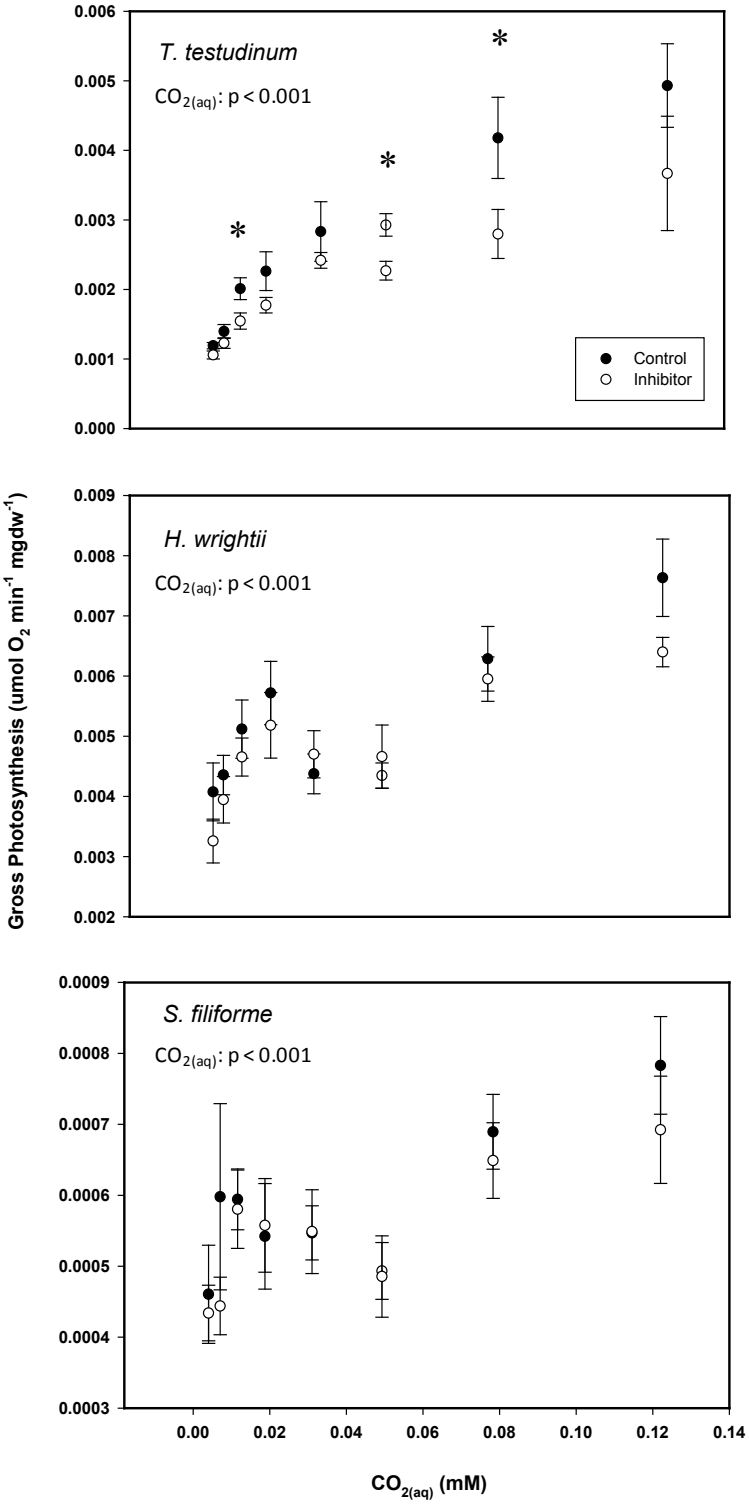
T. testudinum
CO2(aq): p < 0.001
Control
Inhibitor
H. wrightii
CO2(aq): p < 0.001
S. filiforme
CO2(aq): p < 0.001
Gross Photosynthesis (umol O2 min-1 mgdw-1)
CO2(aq) (mM)

FIGURE 2

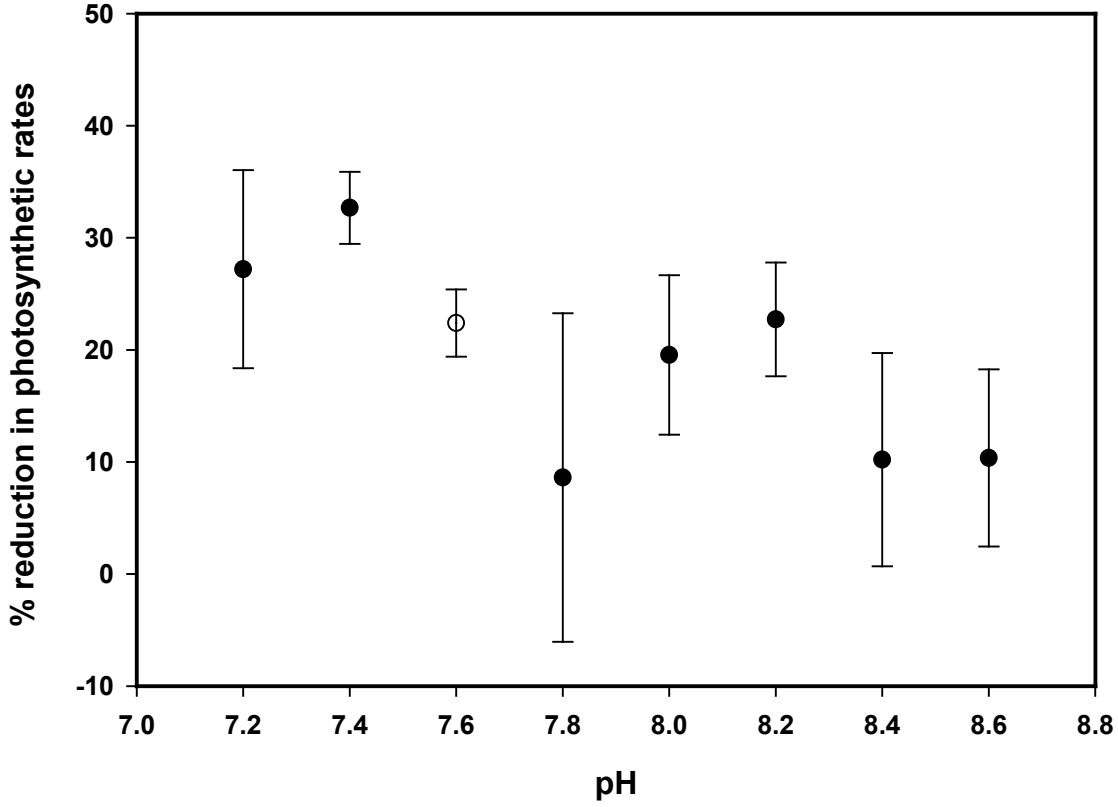
% reduction in photosynthetic rates
50
40
30
20
10
0
-10
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.6
8.8
pH

FIGURE 3.

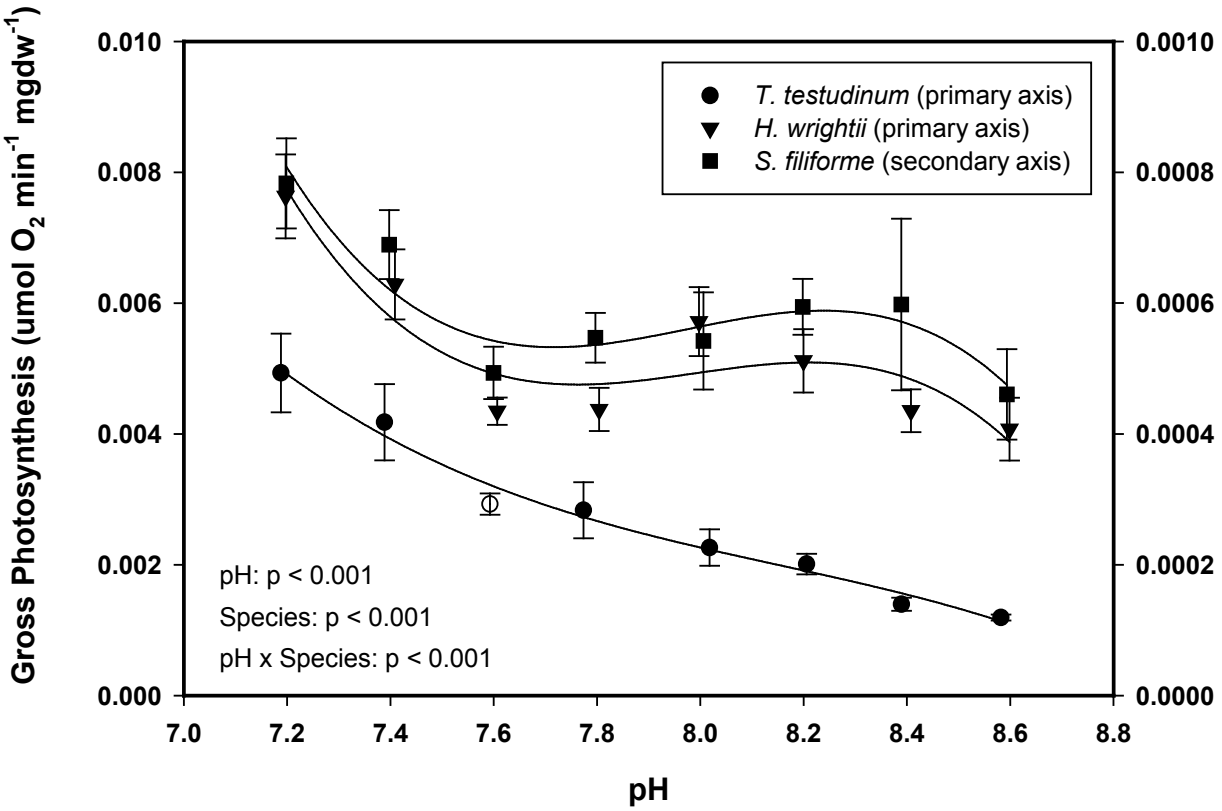
Gross Photosynthesis (umol O_2 min^{-1} $mgdw^{-1}$)
0.010
0.008
0.006
0.004
0.002
0.000
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.6
8.8
pH
T. testudinum (primary axis)
H. wrightii (primary axis)
S. filiforme (secondary axis)
pH: p < 0.001
Species: p < 0.001
pH x Species: p < 0.001

FIGURE 4.

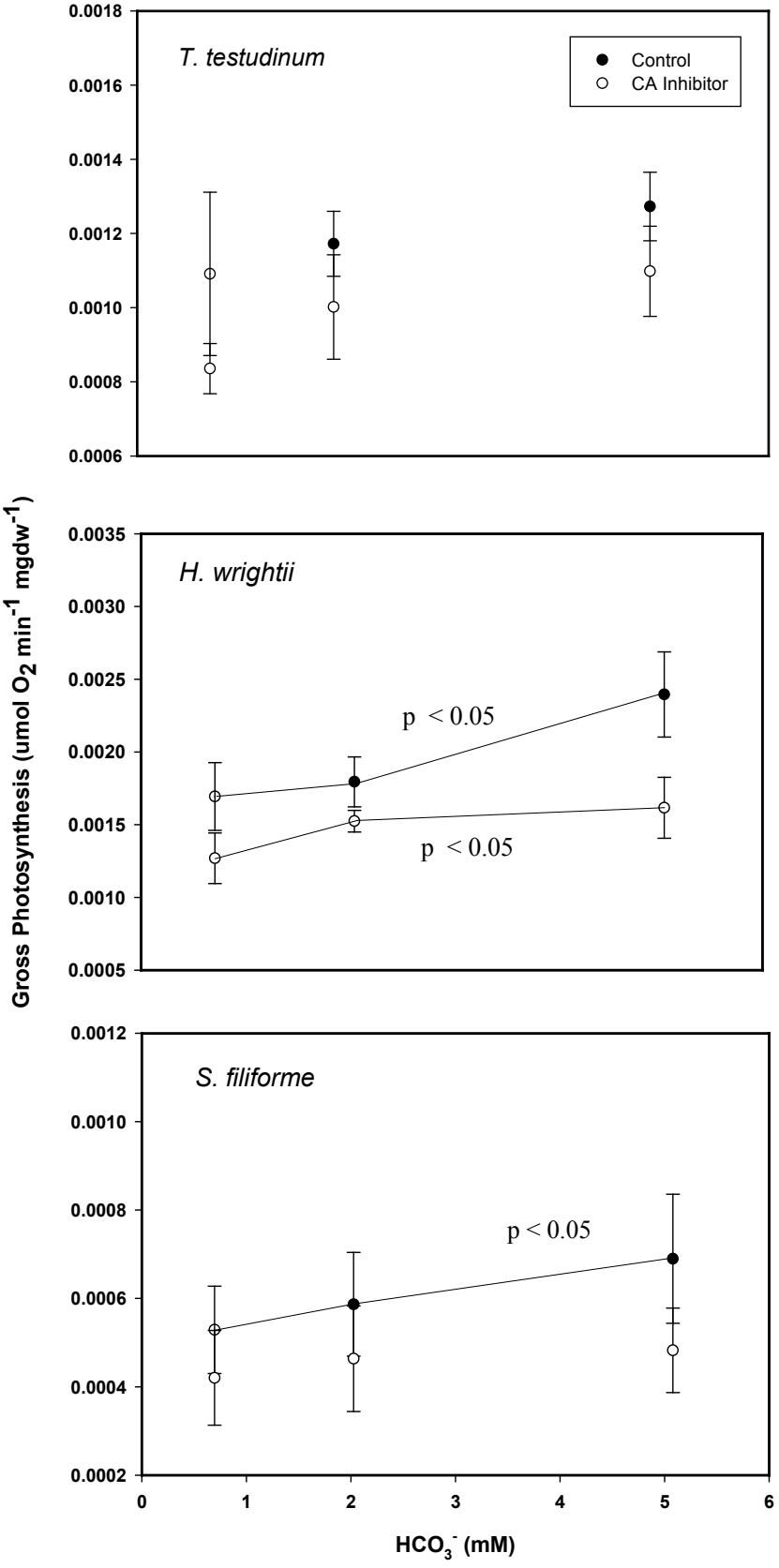
T. testudinum
Control
CA Inhibitor
H. wrightii
p < 0.05
p < 0.05
S. filiforme
p < 0.05
Gross Photosynthesis (umol O2 min-1 mgdw-1)
HCO3- (mM)

Figure 5

CHAPTER II

INTERSPECIFIC VARIATION IN THE ELEMENTAL AND STABLE ISOTOPE CONTENT OF SEAGRASSES IN SOUTH FLORIDA

Campbell JE, Fourqurean JW (2009) Interspecific variation in the elemental and stable isotope content of seagrasses in South Florida. Marine Ecology Progress Series 387:109-123

ABSTRACT

The elemental (C, N and P) and isotopic ($\delta^{13}C$, $\delta^{15}N$) content of leaves of the seagrasses *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* were measured across a 10,000 km^2 survey of the seagrass communities of South Florida in 1999 and 2000. Trends at local and broad spatial scales were compared to examine interspecific variation in the seagrass characteristics often used as ecological indicators. The elemental and stable isotope contents of all species were variable and demonstrated marked interspecific variation. At broad spatial scales mean nitrogen: phosphorus (N:P) ratios were lowest for *T.testudinum* (36:5 ± 1.1) and *S. filiforme* (38:9 ± 1.3), and highest for *H. wrightii* (44:1 ± 1.8). Stable carbon isotope ratios ($\delta^{13}C$) were highest for *S. filiforme* (-6.2‰ ± 0.2‰), intermediate for *T. testudinum* (-8.6‰ ± 0.2‰), and lowest for *H. wrightii* (-10.6‰ ± 0.3‰). Stable nitrogen isotopes ($\delta^{15}N$) were heaviest for *T. testudinum* (2.0‰ ± 0.1‰), and lightest for *H. wrightii* (1.0‰ ± 0.3‰) and *S. filiforme* (1.6‰ ± 0.2‰). Site depth was negatively correlated to $\delta^{13}C$ for all species, while $\delta^{15}N$ was positively correlated to depth for *H. wrightii* and *S. filiforme*. Similar trends were held for local comparisons, suggesting that taxon specific physiological/ecological properties strongly control interspecific variation in elemental and stable isotope content. Temporal trends in $\delta^{13}C$ were measured, and revealed that interspecific variation was displayed throughout the year. This work documents interspecific variation in the nutrient dynamics of three common seagrasses in South Florida, indicating that interpretation of elemental and stable isotope values needs to be species specific.

INTRODUCTION

The elemental and isotopic content of plant biomass can be used to characterize both the nutritional status and environmental conditions of macrophyte communities (Mcmillan et al. 1980; Atkinson and Smith 1983; Farquhar et al. 1989; Duarte 1992; Fourqurean et al. 2005; Fourqurean et al. 2007). These plant parameters, while reflecting the local availabilities of essential resources (nitrogen, phosphorus, carbon, and light), can additionally reflect interspecific variation and the manner in which species interact with local resources. Taxonomic differences in growth rates, life-history strategies, physiology and morphology have the ability to influence resultant nitrogen:phosphorus (N:P) ratios and the stable isotopic content of plant material (Farquhar et al. 1989; Dawson et al. 2002; Agren 2004). While landscape patterns in elemental and isotopic content of submerged plants may be driven by large scale spatial variations in the stable isotopic composition of nutrient pools and the availabilities of nutrients and light, localized interspecific differences have been detected (Fourqurean et al. 2007), and may be attributed to physiological differences amongst sympatric plant species. Prior to using variations in the N:P ratios and stable isotopic compositions of benthic plants for inferring ecosystem processes, it is important to understand the factors which drive these variations at all spatial scales, and how taxonomic differences can be reflected within these parameters.

The N:P ratio of plant material is related to the availability of these elements in the environment relative to plant demand (Duarte 1990). Over landscape scales, spatial gradients in N or P availability are reflected by spatial patterns in plant nutrient content,

as shown for seagrasses growing in coastal marine habitats (Fourqurean et al. 1992a; Fourqurean and Zieman 2002; Fourqurean et al. 2005). Nutrient addition experiments have supported the close link between environmental availabilities and tissue nutrient content, particularly in locations where ambient elemental concentrations are low (Duarte 1990; Ferdie and Fourqurean 2004; Armitage et al. 2005). Other environmental factors can also influence the nutrient content of plant material. For example, increased light availability results in decreased nutrient content of seagrass leaves because of enhanced growth rates leading to the depletion of nutrient sources (Abal et al. 1994). Thus, it has been widely documented that resource availability strongly controls intraspecific variation in the elemental ratios of benthic macrophytes. However, variation between plant species may be driven by factors other than environmental conditions, suggesting that ecological/physiological differences may drive interspecific variation at any particular location. While single species comparisons have been used across a broad range of seagrasses over large spatial scales, localized interspecific comparisons may provide important cues about the ecological differences occurring among sympatric species (Fourqurean et al. 2007).

The stable isotopic content of plant material has provided a powerful tool for the study and assessment of ecological processes. In addition to identifying nutrient sources and processing within ecosystems (Dawson et al. 2002), stable isotopes have aided in food web analysis and the study of energy flow amongst trophic levels (Peterson and Fry 1987). However, the factors regulating the stable isotopic content of primary producers are complex, and require detailed knowledge of spatial, temporal, and taxonomic variation. For example, the stable carbon isotope content of seagrass material is

predominantly controlled by the environmental factors of carbon source, irradiance, and temperature (Durako and Hall 1992; Abal et al. 1994; Grice et al. 1996; Hemminga and Mateo 1996). Spatial and temporal variation in these factors influence the carbon isotopic content of seagrass species across landscape scales (Fourqurean et al. 1997; Fourqurean et al. 2005), highlighting the importance of documenting background variation when applied to food web studies. Numerous studies have detailed the effects of environmental conditions on intraspecific variation in stable isotope parameters. However, interspecific divergence, particularly amongst sympatric seagrasses, has received less attention and may be attributable to physiological/ecological distinctions. Stable isotope comparisons among co-occurring species may reveal important cues pertaining to how specific species process local resources.

Seagrasses fractionate the available pool of inorganic carbon based upon the degree of carbon demand relative to the degree of carbon supply. Experimental evidence has shown that for a given carbon supply, plants grown under high light conditions display increased photosynthetic rates and increased carbon demand, resulting in reduced discrimination against ^{13}C and heavier isotopic signatures (Cooper and Deniro 1989; Durako and Hall 1992). Similarly, carbon isotope signatures ($\delta^{13}C$) increase with decreases in carbon supply (Durako and Sackett 1993). These relationships provide environmental information pertaining to the light and inorganic carbon status of plant material. However, interspecific variation in the mechanisms by which plants process carbon can additionally impact $\delta^{13}C$ values (Farquhar et al. 1989), thus individual plant physiology may contribute to overall variation in stable carbon isotope values. Utilization of bicarbonate (which is isotopically distinct from CO_2) may impact $\delta^{13}C$ ratios

(Hemminga and Mateo 1996), and may further contribute to variation in isotope values. Bicarbonate use is dependent upon species specific seagrass physiology (Invers et al. 1999), thus interspecific differences in stable isotope values may be attributed to the varied physiologies associated with HCO_3^- uptake. Systematic differences in the $\delta^{13}C$ ratios of leaves of co-occurring seagrass species, as documented between the Mediterranean species *Posidonia oceanica* and *Cymodocea nodosa* (Fourqurean et al 2007), suggests fundamental differences in the way that species interact with the available DIC pool.

Seagrass stable nitrogen content additionally provides important information pertaining to the identity of the sources of dissolved inorganic nitrogen (DIN), and the various processes which serve to fractionate the available nutrient pool. For example, as bacterial processing (nitrification, denitrification, and nitrogen fixation) alters the ratio of ^{15}N:^{14}N in the DIN pool, the macrophytic composition of stable nitrogen isotopes is influenced, and thus can be utilized to infer degrees of nitrogen cycling (Peterson and Fry 1987; Dawson et al. 2002). Due to this microbial processing, sewage derived nutrient inputs are isotopically heavy, which can be used to detect possible anthropogenic contributions to the DIN pool.

Similar to carbon isotope discrimination, seagrasses can fractionate the source pool of dissolved inorganic nitrogen upon plant uptake (Fourqurean et al. 2005). The degree of nitrogen fractionation depends upon the size of the DIN pool relative to plant demand (Fourqurean et al. 2005). Seasonal fluctuations in the $\delta^{15}N$ value of seagrass tissues have been attributed to changes in both the isotopic signature of the source pool, and the degree of plant fractionation as a response to seasonal productivities (Fourqurean

et al. 2005). However, altered DIN fractionation among multiple species has yet to be documented.

In this paper, we document interspecific divergence in the elemental and stable isotope content of three seagrass species in South Florida, and explore environmental correlates of these variations across both local and broad spatial scales. Our aim is to better characterize the sources of variation in elemental and isotopic ratios of plants often used as environmental indicators, and highlight the role that ecological/physiological characteristics play in determining species specific values. Seagrass properties (N:P, limitation index, $\delta^{13}C$, and $\delta^{15}N$) were compared across 136 sites in South Florida, representing a 10,000 km^2 survey area of the seagrass beds in the Florida Keys National Marine Sanctuary (FKNMS). We were particularly interested in documenting interspecific variation in the properties of seagrasses co-occurring under similar environmental conditions, within a given site. We hypothesize that the varied life history strategies of benthic macrophytes would be reflected in plant elemental content, with fast-growing, early successional species displaying N:P ratios further removed from ideal values as compared to slow-growing species from the same site. We additionally hypothesize that species-specific differences in carbon uptake strategies and plant physiologies have the ability to strongly regulate stable isotope parameters. Lastly, we hypothesize that fast growing, early successional species may exhibit larger seasonal variation in stable isotope values as a result of increased growth rates and elevated carbon and nitrogen demand. Interspecific comparisons at both local (within site) and broad (amongst all sites) scales allowed us to examine the spatial extent to which taxonomic

variation is important, and its impact on the isotopic and elemental composition of benthic plants.

METHODS

The Florida Keys National Marine Sanctuary (FKNMS) is a shallow-water, marine ecosystem located at the southern tip of the Florida peninsula and comprised of seagrass beds, coral reefs, and mangrove communities (Fig. 1). Seagrass communities in the FKNMS are primarily composed of *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme*. During the summer months of 1999, 80 sites were randomly selected across the FKNMS, and surveyed for seagrass abundance, nutrient content and isotopic composition. A repeat survey was conducted during the summer of 2000 at 56 different randomly generated sites, which only quantified seagrass abundance and nutrient content. Seasonal variation in seagrass isotopic content was additionally assessed through a separate series of quarterly surveys conducted during 1999 and 2000. Within each year, a network of 30 permanent monitoring stations was sampled 4 times (see Fourqurean et al. 2001 for further description). The elemental and isotopic composition of *T. testudinum* has been previously described for this region (Fourqurean et al. 2005). In order to examine interspecific variation, our current study incorporates a portion of those data on the elemental and isotopic content of *T. testudinum*, with new data for the other two seagrass species (*S. filiforme* and *H. wrightii*) common in South Florida.

From the selected random sites, three separate comparative analyses were conducted which spanned various spatial and seasonal scales. One analysis included all data from the 1999 (80 sites) and 2000 (56 sites) surveys, representing comparisons

within the summer season over large spatial scales. Both monospecific (22%) and mixed species (78%) seagrass beds were included. A second analysis included data from the 1999 and 2000 summer surveys, however monospecific sites were excluded, thus interspecific comparisons were solely conducted within multi-species sites where seagrasses co-occurred under similar environmental conditions. Each multi-species site contained two or more seagrass species growing adjacently (< 50m apart), under similar light and depth regimes. Multi-species sites were grouped according to pairwise interspecific comparisons: *Thalassia testudinum*/*Halodule wrightii* (65 sites), *Halodule wrightii*/*Syringodium filiforme* (38 sites), and *Thalassia testudinum*/*Syringodium filiforme* (75 sites). The third analysis similarly conducted within-site interspecific comparisons, however only utilized data from the 30 permanent monitoring sites, allowing for comparisons of intra-annual variability between species pairs. Within this network, the number of sites for interspecific comparison varied depending upon season; *T. testudinum*/*H. wrightii* (4-7 sites), *H. wrightii*/*S.filiforme* (5-8 sites), *T. testudinum*/*S. filiforme* (19-22 sites).

For all surveys, at each sampling site, short shoots of each seagrass species present were haphazardly collected along a 50m transect. When available, 6 shoots of *Thalassia testudinum*, 30 shoots of *Syringodium filiforme*, and 40 shoots of *Halodule wrightii* were harvested, placed on ice, and transported back to the lab. The less robust seagrass species required higher collection amounts to ensure enough biomass was available for all elemental and isotopic analyses. Seagrass leaves were separated according to species, cleaned of epiphytes through gentle scraping with a razor blade, and cut from their respective short shoots. Leaves were then dried to a constant weight at

80^{o}C, ground to a fine powder with a mortar and pestle, and analyzed in duplicate for C and N content using a CHN analyzer (Fourqurean et al. 2005). Phosphorus content was determined through dry oxidation, acid hydrolysis extraction followed by a colorimetric analysis (Fourqurean et al. 1992a). Elemental ratios were calculated on a mole:mole basis.

All isotopic analyses were measured using standard elemental analyzer isotope ratio mass spectrometer procedures. The elemental analyzer was used to combust all organic material and subsequently reduce the formed gasses into N_2 and CO_2, which were measured on a Finnigan MAT Delta C IRMS in a continuous flow mode. The samples' isotopic ratios (R) are reported in the standard delta notation (‰): δ (‰) = $[(R_{sample}/R_{standard})-1] \times 1000$. These results are presented with respect to the international standards of atmospheric nitrogen (AIR,N_2) and Vienna Pee Dee belemnite (V-PDB) for carbon. Analytical reproducibility of the reported δ values, based on sample replicates, was better than ± 0.08‰ for carbon and ± 0.2‰ for nitrogen. Care was taken to remove all visible carbonate material from the surface of the leaves. As a test of the efficacy of our cleaning we acidified a subset of seagrass samples with the most enriched $\delta^{13}C$ values to drive off any remaining carbonate material, and then determined the $\delta^{13}C$ of this decalcified material. The differences in $\delta^{13}C$ between acidified and unacidified samples were small (< 0.3 ‰ on average).

The distributions of all seagrass elemental and isotopic parameters were checked for normality using the Kolmogorov-Smirov test (α=0.05). Standard linear regression was used to test the strength of the relationship between stable isotope variables ($\delta^{13}C$, $\delta^{15}N$) and site depth. Relationships among elemental and isotopic ratios were additionally

assessed for all species, across all sites sampled in 1999, using non-parametric correlations (Spearman's ρ). Interspecific Sanctuary-wide species-specific differences in seagrass N:P ratios and isotopic values were assessed using a single factor analysis of variance (ANOVA). Upon detecting significance, post-hoc analysis was conducted using either a Tukey's HSD for equal variances, or a Dunnett's T3 test for unequal variances (significance $p < 0.05$). In addition to testing N:P ratios, a Limitation Index (L.I. = |30-N:P|) was calculated to quantify the degree of divergence from the ideal ca. 30:1 "Seagrass Redfield Ratio" identified by Atkinson and Smith (1983) and Duarte (1990). Larger L.I. values indicate greater degrees of nutrient limitation. Such a calculation is necessary because across the landscape of the FKNMS there are both N- and P-limited regions (Fourqurean et al 2005).

To conduct within-site interspecific comparisons, differences in N:P ratios, L.I., $\delta^{13}C$, and $\delta^{15}N$ were tested with a pair-wise Students t-test, significance $p < 0.05$. To describe temporal variation in seagrass isotopic content for species that co-occurred at the 30 permanent stations, we fit a sine model of the form $y = \text{mean} + \alpha[\sin(\text{time} + \Phi)]$, where α is the amplitude of a sine wave, and Φ is a phase angle (we used time values in radians for both time and Φ, where 2π radians = 365d), to a seasonal time series using an iterative nonlinear curve fitting regression. Confidence intervals (95%) of model parameters were compared to test for significant differences in seasonal variation. We were interested in using the non-linear regression as a means of testing interspecific divergence in the mean and amplitude of seasonal seagrass $\delta^{13}C$ variation.

RESULTS

Interspecific variation in seagrass elemental and isotopic composition at broad scales across the FKNMS

In 1999, *Thalassia testudinum* occurred at 78 of the randomly selected sites (98%), while *Halodule wrightii* and *Syringodium filiforme* occurred at 31 sites (39%) and 37 sites (46%) respectively. In 2000, *T. testudinum* occurred at 56 sites (100%), while *H. wrightii* and *S. filiforme* occurred at 39 sites (70%) and 40 sites (71%) respectively. Nitrogen, phosphorus and carbon content varied within each species, across all sanctuary sites in both years (Table 1), however they did not vary significantly between years. In both years, coefficients of variation show that leaf phosphorus content (CV=0.28-0.37) displayed greater variation in all species compared to nitrogen (CV=0.15-0.25) and carbon content (CV=0.06-0.12). The N:P frequency distribution for *T. testudinum* was slightly skewed towards higher values (Fig. 2). Comparatively, *H. wrightii* displayed a normal distribution with the highest mean N:P ratios (44.1 ± 1.8), which were reflected in the highest mean L.I. of all species (15.3 ± 1.7, Fig. 3, Table 1). *Syringodium filiforme* displayed an intermediate N:P ratio of 38.9 ± 1.3, which was normally distributed, and an average limitation index value of 10.9 ± 1.1. *Thalassia testudinum* displayed the lowest mean N:P ratio (36.5 ± 1.1), and the lowest mean L.I. value (9.6 ± 0.9) of the species. Mean *T. testudinum* N:P ratio and L.I. values were significantly lower than those of *Halodule wrightii*, yet similar to *Syringodium filiforme* (Table 1). Mean *H. wrightii* N:P ratio and L.I. values were similar to *S. filiforme* (ANOVA, $F = 7.89$, $p = 0.07$; ANOVA, $F = 6.25$, $p = 0.09$).

Halodule wrightii displayed the lowest $\delta^{13}C$ values of all species, with a mean of -10.6 ‰ ± 0.3‰, a range of -13.2‰ to -7.8‰, and a normal distribution (Fig. 4). *Halodule wrightii* $\delta^{13}C$ values were significantly lower than both *Thalassia testudinum* and *Syringodium filiforme* (Table 1; ANOVA, $F = 53.5$, $p < 0.001$). *Syringodium filiforme* displayed the highest $\delta^{13}C$ values with a mean of -6.2‰ ± 0.2‰, a range of -3.5‰ to -8.4‰, and a normal distribution. The $\delta^{13}C$ values of *S. filiforme* were significantly higher than those of *T. testudinum* (ANOVA, $F = 53.5$, $p < 0.001$). *Thalassia testudinum* displayed intermediate $\delta^{13}C$ values with a mean of -8.6‰ ± 0.2‰, a range of -13.0‰ to -5.3‰, and values that were normally distributed.

Stable nitrogen isotope values varied by 7.7‰, 7.5‰, and 6.3‰ for *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* respectively (Table 1). *Thalassia testudinum* displayed the highest $\delta^{15}N$ values of all species, with mean of 2.0‰ ± 0.2‰ and a normal distribution (Fig. 5). *Thalassia testudinum* displayed $\delta^{15}N$ values which were higher than those of *H. wrightii* (Table 1; ANOVA, $F = 5.08$, $p < 0.01$), yet similar to *S. filiforme* (ANOVA, $F = 5.08$,$p = 0.45$). *Halodule wrightii* displayed the lowest values, with a mean of 1.0‰ ± 0.3‰ and a normal distribution. *Halodule wrightii* $\delta^{15}N$ values were similar to those of *S. filiforme* (ANOVA, $F = 5.08$,$p = 0.18$). *Syringodium filiforme* displayed intermediate values, with a mean of 1.6‰ ± 0.3‰ and a normal distribution.

Across FKNMS, elemental and isotopic ratios of all three seagrass species were correlated with site depth (Table 2, Fig. 6). The $\delta^{13}C$ and N:P ratios were negatively correlated to site depth for all species, and displayed similar variation with depth amongst species. The $\delta^{15}N$ ratio was positively correlated with site depth for *Halodule wrightii*

and *Syringodium filiforme*, yet there was no correlation for *Thalassia testudinum* (Fig. 6). The variation in $\delta^{15}N$ with depth was similar between *H. wrightii* and *S. filiforme*, and further analysis revealed that negative correlations between %N and $\delta^{15}N$ were restricted to the deeper offshore locations (>4.3m). *Syringodium filiforme* was the only species for which $\delta^{13}C$ was correlated to $\delta^{15}N$. Correlations between elemental and isotopic ratios were mixed depending upon species. In *T. testudinum*, N:P was positively correlated to $\delta^{13}C$, yet uncorrelated to $\delta^{15}N$. *Syringodium filiforme* showed a negative correlation between N:P and $\delta^{15}N$, yet no correlation between N:P and $\delta^{13}C$. In *H. wrightii*, N:P was both negatively correlated to $\delta^{15}N$, and positively correlated to $\delta^{13}C$. L.I. values were negatively correlated to both site depth and $\delta^{15}N$ for *H. wrightii* and *S. filiforme*, *T. testudinum* showed no correlation amongst these parameters. For all species L.I. was positively correlated to $\delta^{13}C$.

Interspecific variation in seagrass elemental and isotopic composition at local scales

Of the sites which contained both *Thalassia testudinum* and *Halodule wrightii* (65 sites), N:P ratios and L.I. values were significantly higher for *H. wrightii* ($p < 0.001$; Fig. 7). When averaged for both years, *T. testudinum* had an N:P ratio and an L.I. value of 35.9 ± 1.4 and 8.8 ± 1.1 respectively at sites where it co-occurred with *H. wrightii*, while *H. wrightii* had an N:P ratio and an L.I. value of 44.5 ± 1.9 and 15.8 ± 1.8 at the same sites. When growing in similar locations, significant differences were additionally detected in $\delta^{13}C$ signatures, with *T. testudinum* (-8.9 ± 0.4) displaying isotopically heavier values than *H. wrightii* (-10.4 ± 0.3) ($p < 0.001$)(Fig. 8). There were no

consistent, statistically significant differences in $\delta^{15}N$ between these two species at sites where they co-occurred.

Halodule wrightii and *Syringodium filiforme* co-occurred at 38 sites, and did not differ in either N:P ratios or L.I. (Fig. 7). For both sampling years, N:P ratios and L.I. were 43.9 ± 2.6 and 15.8 ± 2.2 respectively for *H. wrightii*, and 42.3 ± 2.2 and 14.2 ± 1.9 respectively for *S. filiforme*. Significant differences were detected in $\delta^{13}C$ signatures, with *H. wrightii* displaying a value of -10.1‰ ± 0.3‰, as compared to the isotopically heavy *S. filiforme* (-5.7‰ ± 0.3‰) ($p < .001$)(Fig. 8). There were no consistent, statistical differences in $\delta^{15}N$ between *H. wrightii* and *S. filiforme* at sites where they co-occurred.

Thalassia testudinum and *Syringodium filiforme* co-occurred at 75 sites, and displayed significant differences in both N:P ratios and L.I. at those sites ($p < 0.001$, $p < 0.01$ respectively). For both years, *T. testudinum* displayed an N:P ratio and L.I. value of 34.2 ± 1.1 and 7.4 ± 0.9, respectively. While *S. filiforme* displayed an N:P ratio and L.I. of 38.8 ± 1.4 and 10.9 ± 1.2, respectively (Fig. 7). Additionally, $\delta^{13}C$ signatures were statistically distinct, with *T. testudinum* (-8.8‰ ± 0.2‰) displaying isotopically lighter values than *S. filiforme* (-6.3‰ ± 0.2‰) ($p < 0.001$) (Fig. 8). There were no statistical differences in $\delta^{15}N$ between *T. testudinum* and *S. filiforme* at sites where they co-occurred.

Temporal variation in seagrass isotopic content at local scales

There was marked seasonal variation in the $\delta^{13}C$ values of *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* (Fig. 9). $\delta^{13}C$ values for all three species were heaviest in the summer and fall months, and lightest in the winter months. The sine

models described 51-73% of the variation in $\delta^{13}C$ values (Table 3). Parameter estimates for α and Φ were not significantly different between species comparisons, indicating no interspecific differences in the degree of seasonal variation, nor the seasonal timing of $\delta^{13}C$ values. Mean $\delta^{13}C$ values of the sine models were significantly different for all species comparisons, indicating that the interspecific differences we documented above with our FKNMS-wide summer surveys were maintained throughout the year. Seasonal trends in $\delta^{15}N$ values were not detected for any species.

DISCUSSION

The elemental and isotopic leaf content of the seagrasses *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* displayed marked interspecific variation at both local and broad spatial scales within the Florida Keys National Marine Sanctuary. Both within and between species variations were nonrandom, and demonstrated both the effects of environmental parameters and species-specific physiologies on the elemental and isotopic content of seagrass tissues. This study highlights the importance of seagrass species identity in the evaluation of plant nutrient and isotopic data for coastal monitoring efforts and food web analyses.

Relationships between elemental ratios, isotopic ratios, and water depth across the FKNMS

Significant differences in $\delta^{13}C$ were detected amongst all three seagrass species. The lightest $\delta^{13}C$ values were displayed by *Halodule wrightii*, while *Thalassia testudinum* and *Syringodium filiforme* displayed the intermediate and heaviest values respectively. Similarly, Lepoint et al. (2008) found that *Syringodium isoetifolium* was

more enriched in ^{13}C compared to *Halodule* sp. and *Thalassia hemprichii* in Mozambique. The heaviest $\delta^{15}N$ was displayed by *T. testudinum*, which was significantly more positive than *H. wrightii*. The $\delta^{15}N$ value for *S. filiforme* was not statistically distinct from the other species. Large scale trends in the stable isotopic content of various seagrasses may provide information pertaining to physiological and ecological properties of each species. However, data at this large scale should be interpreted with caution, as trends may be confounded by spatial variations in: (1) the distribution of various seagrass species and (2) environmental conditions.

All three seagrass species showed significant correlations between $\delta^{13}C$ values and site depth across the large spatial scale of the sanctuary (Fig. 6). Because light availability generally decreases with depth in the sea, this suggests that for each species, light plays an important role in regulating the $\delta^{13}C$ content of seagrass tissues, as demonstrated in both laboratory and field studies (Durako and Hall 1992; Abal et al. 1994; Grice et al. 1996). This is likely because of reduced photosynthetic discrimination against the heavier isotope, leading to increased $\delta^{13}C$ values at high light levels. Within a species, isotopically heavy values may indicate the possibility of photosynthetic carbon limitation during periods of high irradiance (Fourqurean et al. 2005). Field studies have shown that the inverse relationship between light and depth are reflected in the $\delta^{13}C$ ratio of seagrass tissues elsewhere (Cooper and Deniro 1989; Lepoint et al. 2003; Fourqurean et al. 2007). In our study, $\delta^{13}C$ values decrease by 0.40, 0.29, and 0.25 ‰ m^{-1} in depth for *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme* respectively. We do not think that the $\delta^{13}C$-depth relationships are a function of variations in the isotopic signature of the DIC pool, as has been observed in regions where mineralization of

organic matter from C_3 mangroves causes isotopically depleted DIC that is incorporated into seagrass tissues (Fry and Sherr 1984; Fleming et al. 1990; Lin et al. 1991). The FKNMS sites that are in close proximity to terrestrial DIC sources display the heaviest $\delta^{13}C$ values, contrary to what would be expected if C_3 material was impacting the isotopic value of the DIC pool available to the seagrass community.

The $\delta^{15}N$ value of seagrass leaf material can be controlled by numerous factors. In addition to alterations in the isotopic composition of the source nitrogen pool, the relationship between plant nutrient demand and environmental availability represents a strong determinant of leaf isotope ratios. Reduced light as depth increases could, by reducing leaf growth rates and demand for N, influence leaf $\delta^{15}N$ as depressed demand increases discrimination against the heavier isotope. However, the net change in leaf $\delta^{15}N$ with increasing depth should be a consequence of the magnitude of the reduction in N demand relative to the changes in N availability. For instance, in cases where N availability remains constant with increasing depth, we would expect a decrease in leaf $\delta^{15}N$ with increasing depth due to a depressed demand for N and higher discrimination against ^{15}N. Alternatively, cases in which leaf $\delta^{15}N$ shows little change with depth may represent scenarios whereby both N demand and N availability concurrently decrease with depth, resulting in little alteration to ^{15}N discrimination. Working with other species of seagrasses, Grice et al. (1996), Lepoint et al. (2003), and Fourqurean et al. (2007) all observed no significant change in leaf $\delta^{15}N$ across large depth and light gradients, although they did not investigate the causal mechanisms for those observations. Lastly, if nitrogen availability decreases dramatically with increasing depth, we could, despite reductions in N demand, find higher leaf $\delta^{15}N$ values as depth increases. We believe this

to be the case in our study, as the observed relationships between $\delta^{15}N$ and depth are a consequence of the distribution of deep-water sites within a landscape of variable nitrogen availability. The leaf $\delta^{15}N$ value of both *Halodule wrightii* and *Syringodium filiforme* was positively correlated to site depth, and negatively correlated to both N:P ratios and L.I. Such correlations suggest that as nitrogen becomes less available and nutrient limitation increases, these seagrasses reduce fractionation of the available DIN pool. Additionally, the negative correlation between %N and $\delta^{15}N$ was only significant for the deeper sites under similar light regimes, further indicating that environmental availability was driving the relationship between depth and $\delta^{15}N$ and that the reduced DIN discrimination in *H. wrightii* and *S. filiforme* increasingly occurs at deeper offshore locations, where primary production is N-limited (Fourqurean and Zieman 2002; Ferdie and Fourqurean 2004; Fourqurean et al. 2005). The relationship between depth and $\delta^{15}N$, however, was not held for *Thalassia testudinum* which exhibited no correlation between $\delta^{15}N$ values and site depth or N:P ratios. Thus, these correlations were only evident for the fastest growing seagrasses (*H. wrightii* and *S. filiforme*), which may utilize nutrient resources more rapidly than *T. testudinum*, leading to decreases in DIN fractionation and higher L.I. compared to the slower-growing *T. testudinum*. Further experimentation is needed to detail these processes.

The fact that interspecific differences in stable carbon isotopes remain statistically distinct across large spatial scales may imply that seagrass physiology plays a dominant role in determining interspecific variation in $\delta^{13}C$. Average values for *Halodule wrightii* were 2.0 ‰ lighter than *Thalassia testudinum*, and 4.5 ‰ lighter than *Syringodium filiforme*. Such differences might be attributed to the varied mechanisms of bicarbonate

acquisition and internal carbon recycling within marine macrophytes (Fry and Sherr 1984; Fry et al. 1985; Hemminga and Mateo 1996; Fourqurean et al. 2007), or caused by morphological variations in leaves (Lepoint et al. 2008). To various degrees, seagrass photosynthetic carbon demand may be met via a combination of diffusive CO_2 transport, and active HCO_3^- import (Invers et al. 1999; Invers et al. 2001). While dissolved CO_2 has an isotopic carbon signature of ca. -9 ‰, HCO_3^- in marine waters has an isotopic signature of 0 ‰. Preferential fixation of bicarbonate from the inorganic carbon pool should result in seagrass tissue which is enriched in ^{13}C (Hemminga and Mateo 1996). Interspecific variation in seagrass $\delta^{13}C$ values may reflect previously documented interspecific variation in bicarbonate acquisition mechanisms, as some species display enhanced HCO_3^- utilization efficiencies (Bjork et al. 1997; Invers et al. 1999; Schwarz et al. 2000; Uku et al. 2005). Our field data suggest that *S. filiforme* may rely more on HCO_3^- as a carbon source than *H. wrightii* or *T. testudinum*, an hypothesis that should be explored.

Internal recycling of CO_2 has been proposed as an alternate mechanism which can influence the stable carbon isotope value of benthic macrophytes (Cooper 1989; Abal et al. 1994; Grice et al. 1996). Seagrass species which have enhanced lacunal volume may display increased recycling of internal carbon pools, and reduced isotopic discrimination. As internal recycling of CO_2 reduces the degree of carbon back-diffusion, RUBISCO operates in an increasingly closed environment, converting all inorganic carbon into organic products, resulting in isotopically heavier $\delta^{13}C$ signatures (Sharkey and Berry 1985). Internal recycling may therefore serve to complicate the relationship between the $\delta^{13}C$ of source DIC, and the $\delta^{13}C$ of seagrass tissues. Our data are consistent with more

efficient internal recycling of CO_2 in *Syringodium filiforme* than *Halodule wrightii* or *Thalassia testudinum*. It is evident that the factors contributing to the characteristic $\delta^{13}C$ value of specific seagrass species are complex, and require studies focused on detailing the contributions that carbon acquisition mechanisms, seagrass lacunal volume, and rates of CO_2 recycling make to the overall isotopic signature.

Interspecific variation in $\delta^{15}N$ across FKNMS reveal significant differences between *Thalassia testudinum* and *Halodule wrightii*, however it remains unclear whether this trend is due to differences in the degree of isotope fractionation among species, or due to spatial trends in the isotopic composition of source DIN and seagrass abundance. Across a large spatial scale, *T. testudinum* was isotopically heavier than *H. wrightii*, indicating decreased fractionation amongst nitrogen isotopes. Successional studies have demonstrated *T. testudinum* as a climax species with the ability to thrive in nutrient poor environments, and *H. wrightii* as an early successional species thriving under more eutrophic conditions (Fourqurean et al. 1995). Across large spatial scales, *T. testudinum* may monospecifically occupy the most oligotrophic locations with the lowest DIN pools, resulting in decreased fractionation of the nitrogen isotope, accounting for heavier $\delta^{15}N$ values; *H. wrightii*, occupying areas with higher DIN pools may have higher rates of fractionation, lowering $\delta^{15}N$ values. Such results are not contradictory to previous findings concerning the correlation between $\delta^{15}N$ and depth. Despite *H. wrightii* displaying decreased $\delta^{15}N$ fractionation with depth, overall this species remains isotopically lighter than *T. testudinum* because it is excluded from the highly oligotrophic deeper waters solely occupied by *T. testudinum*.

Elemental comparisons across FKNMS reveal that *Thalassia testudinum* had significantly lower N:P ratios and L.I. than *Halodule wrightii* ($p < 0.01$), while *Syringodium filiforme* was not statistically distinct from either *T. testudinum* or *H. wrightii*. Overall, intraspecific variation at this scale is attributable to the balance between the availability nutrient resources (N and P) and rates of seagrass productivity (Fourqurean et al. 2005). However interspecific differences in the N:P ratios of *T. testudinum* and *H. wrightii* may result from life history differences between these 2 species. The early successional status and higher growth rates of *H. wrightii* may account for increased deviation from seagrass Redfield N:P stoichiometry. Nutrient demand is higher for *H. wrightii* than for *T. testudinum* (Fourqurean et al. 1992b), thus despite possibly being limited to sites elevated in nutrient concentrations, the fast growth rates of *H. wrightii* still produce N:P ratios which are drastically altered from Seagrass Redfield values. Ecologically, *T. testudinum* is a late successional species, and the life history strategy of reduced growth rates may allow *T. testudinum* to produce biomass with N:P ratios closer to the ideal Redfield value of 30:1. The extensive investment in underground biomass (root/rhizome complex) may additionally allow *T. testudinum* to exploit sediment nutrient pools unavailable to other species, thus bringing stoichiometric ratios closer to 30:1. However, across landscape scales, elemental variation because of life history differences are difficult to separate from elemental variation that result from spatial trends in nutrients, light, and seagrass abundance. For example, if *T. testudinum* were relatively more abundant in deeper, lower light environments, then lower productivities and reduced N:P ratios may be attributable to this spatial environmental factor, and not species-specific physiological/ecological properties.

Interspecific variation at local scales within the FKNMS

Within site, local comparisons between species control for spatial gradients in abiotic factors (light and nutrients), hence differences in elemental and isotopic compositions do not reflect environmental variation, and may be attributed to differences in species specific physiological and ecological properties. Congruence or discordance of local trends with broad spatial trends reveals whether interspecific variation is the result of physiological attributes or wide ranging abiotic variation. We find that at the local scale, interspecific trends in stable carbon isotopic content and elemental ratios are generally held, while trends in stable nitrogen isotopic content are not consistent with the trends observed at broad spatial scales.

Local-scale interspecific trends in $\delta^{13}C$ agree with broad scale trends, revealing that species specific physiological attributes are dominant factors in controlling taxon specific $\delta^{13}C$ values. At the same location, *Halodule wrightii* was significantly lighter than both *Thalassia testudinum* and *Syringodium filiforme*, and *T. testudinum* was significantly lighter than *S. filiforme*, yet heavier than *H. wrightii* (Fig. 8), suggesting that the interspecific trends at the broad scale of the FKNMS are functions of the physiological attributes of carbon acquisition. Utilizing this model, *H. wrightii* may represent a seagrass species which, while displaying HCO_3^- use, exhibits high rates of carbon back diffusion (low CO_2 recycling), and thus fails to fix a large portion of incorporated carbon, allowing for increased isotopic discrimination. Conversely, *S. filiforme* may represent a species which displays reduced carbon back diffusion (high CO_2 recycling), and thus fixes a large majority of imported carbon. The apparent dependence of seagrass $\delta^{13}C$ on specific carbon acquisition properties has terrestrial

analogues, as seen in the $\delta^{13}C$ variation between C_3 and C_4 plants. In addition to utilizing PEP carboxylase to fix CO_2, which discriminates less against ^{13}C than RUBISCO, C_4 plants limit carbon diffusion out of the leaves with morphological adaptations (e.g. bundle sheaths), and variations in the rates of CO_2 back diffusion have been noted to impact the $\delta^{13}C$ values of C_4 vegetation (Farquhar et al. 1989). While seagrasses are all C_3 plants (Beer and Wetzel 1982), the degree of bicarbonate use, extent of internal recycling, and specific leaf morphology (Lepoint et al. 2008) are likely to play analogous roles in explaining interspecific variation in the $\delta^{13}C$ value of marine plants.

Local-scale interspecific trends in stable nitrogen isotope values displayed non-significant differences amongst all seagrass species. The significant differences observed between *Thalassia testudinum* and *Halodule wrightii* at the broad-scale were not observed within sites, indicating that $\delta^{15}N$ trends across FKNMS were predominantly a result of spatial variation in seagrass distribution and DIN pools, as opposed to interspecific physiological differences in nitrogen uptake and fractionation. However, we note that the reduced sampling effort of the within site comparisons may have limited our ability to detect a significant difference in $\delta^{15}N$ between *T. testudinum* and *H. wrightii*, warranting future studies.

Interspecific variation in elemental ratios revealed that, similar to broad scale patterns, *Thalassia testudinum* displayed significantly lower N:P ratios than *Halodule wrightii* when growing at the same location. At the local scale, *T. testudinum* also had significantly lower N:P ratios than *S. filiforme*, previously undocumented at broader scales. There remained no difference in N:P ratios between *H. wrightii* and *S. filiforme*. By removing spatial variation in environmental variables and seagrass distribution,

interspecific differences in elemental composition reflect important differences in the ecology of these seagrass species. When co-occurring with *T. testudinum*, and exposed to similar nutrient and light conditions, rapidly growing early successional species (*H. wrightii* and S. *filiforme*) are further removed from Seagrass Redfield stoichiometry, indicating heavily nutrient limited growth (particularly with respect to phosphorus). The slower growth rates of *T. testudinum* may allow for reduced nutrient limited growth, and an enhanced ability to thrive under oligotrophic conditions. The altered elemental ratios of co-occurring seagrasses further suggests that nutrient limitation needs to be viewed in respect to a specific primary producer, and may not be applicable to other species within the same location.

Temporal variation in seagrass isotope content

As previously documented for *Thalassia testudinum* (Fourqurean et al. 2005) the $\delta^{13}C$ of *Halodule wrightii* and *Syringodium filiforme* fluctuate seasonally. Interspecific variation in seagrass $\delta^{13}C$ value was not limited to the summer conditions of high productivity, as we would expect if differential CO_2 acquisition were leading to different amounts of isotopic discrimination during summer periods of maximum photosynthesis. Interspecific differences in $\delta^{13}C$ were maintained throughout the year, with peaks during the highly productive summer periods and lows during the less productive winter periods. The constant interspecific variation suggests that species specific $\delta^{13}C$ values are a result of physiologically based traits, and are unlikely the result of altered or adaptive carbon acquisition mechanisms during the summer months. Thus, despite seasonal variation, seagrass $\delta^{13}C$ values remain significantly distinct throughout the year, and maintain the

similar trends (*S. filiforme* > *T. testudinum* > *H. wrighii*) observed during the summer surveys.

Seasonal variation in seagrass $\delta^{13}C$ values have been correlated to seasonal patterns in productivity, governed both by light availability and temperature (Fourqurean et al. 2001; Fourqurean et al. 2005; Fourqurean et al. 2007). Drawdown of CO_2 pools during the summer months, combined with limited CO_2 diffusion rates, may decrease the degree of fractionation displayed by RUBISCO. Similar seasonal trends have been noted for a number of phytoplankton studies, and may indicate carbon limitation during periods of high productivity (Fogel et al. 1992). Alternately, it is possible that increased summer productivities may enhance bicarbonate use within all seagrass species, elevating $\delta^{13}C$ values and mitigating carbon limited photosynthesis. The amplitudes of the sine models were not statistically distinct among species, thus there was no difference in the degree of seasonal variation. These findings contrasted with our original hypotheses, which expected the fastest growing seagrass species (*H. wrightii* and *S. filiforme*) to exhibit the greatest amplitude in seasonal variation, suggesting that while taxonomic differences strongly influence the mean $\delta^{13}C$ value, seasonal environmental factors strongly control annual variation within each species. There were no interspecific differences in Φ, and therefore the timing of the seasonal response (summer peaks and winter lows) amongst species. It is evident that while seasonal responses are not different in all three seagrass species, their mean $\delta^{13}C$ value remains markedly distinct throughout the annual cycle.

Seasonal variation in seagrass $\delta^{15}N$ was undetected for all species. Seasonal cycles in stable nitrogen isotope content have been previously documented for *Thalassia testudinum* in South Florida (Fourqurean et al. 2005), thus the lack of a seasonal pattern

in our study may be due to an insufficient sampling size for this particular isotope parameter.

The elemental and stable isotopic content of seagrass species in South Florida displays considerable interspecific variation at both broad and local spatial scales. Here we present the first documentation of species-specific variation in South Florida; by which carbon isotopic values may be associated with physiological differences in carbon acquisition and elemental ratios may be associated with ecological differences in seagrass growth rates. As such, the use of these parameters as indicators of ecosystem properties needs to be carefully considered for the species of marine plant being studied. In addition to previously documented spatial and temporal variation, taxonomic differences in isotopic and elemental content can introduce additional variation of significant magnitude. Interspecific differences are non-random, thus understanding how seagrass species differ in isotopic and elemental content will aid in the interpretation of studies which examine the food web and nutrient dynamics of multi-species seagrass ecosystems.

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Table 1: Elemental and stable isotopic composition of seagrass leaves collected across FKNMS in 1999 and 2000 (L.I. = Limitation Index). Superscripts on means within a column identify significantly different groups (post-hoc tests, $p<0.05$)

	$\delta^{15}N$	$\delta^{13}C$	%C (% dry wt)	%N (% dry wt)	%P (% dry wt)	C:N	C:P	N:P	L.I.
Thalassia testudinum									
Mean	2.0 ^{A}	-8.6 ^{A}	39.2 ^{A}	1.9 ^{A}	0.13 ^{A}	24.1 ^{A}	870.8 ^{A}	36.5 ^{A}	9.6 ^{A}
n	78	78	134	134	134	134	134	134	134
SE	0.15	0.20	0.20	0.02	0.00	0.31	26.28	1.07	0.88
C.V.	0.70	0.20	0.06	0.15	0.28	0.15	0.35	0.34	1.07
Median	1.8	-8.3	39.6	1.9	0.13	24.0	783.7	34.4	6.2
Minimum	-2.2	-13.0	31.1	1.4	0.06	17.1	500.3	17.1	0.2
Maximum	5.4	-5.3	43.2	2.6	0.22	33.9	1902.3	76.5	46.5
Halodule wrightii									
Mean	1.0 ^{B}	-10.6 ^{B}	43.4 ^{B}	2.3 ^{B}	0.13 ^{A}	22.7 ^{A}	1014.1 ^{B}	44.1 ^{B}	15.3 ^{B}
n	31	31	70	70	70	70	70	70	70
SE	0.29	0.28	0.27	0.05	0.01	0.44	51.76	1.82	1.67
C.V.	1.70	0.15	0.05	0.17	0.37	0.16	0.43	0.35	0.91
Median	0.9	-10.8	43.9	2.26	0.12	22.5	890.9	39.7	9.7
Minimum	-3.5	-13.2	35.0	1.48	0.05	16.7	472.3	23.7	0.3
Maximum	4.0	-7.8	46.3	3.18	0.25	33.9	2572.2	94.9	64.9
Syringodium filiforme									
Mean	1.6 ^{AB}	-6.2 ^{C}	38.9 ^{A}	2.1 ^{C}	0.13 ^{A}	22.8 ^{A}	866.0 ^{A}	38.9 ^{AB}	10.9 ^{AB}
n	37	37	77	77	77	77	77	77	77
SE	0.25	0.21	0.55	0.06	0.00	0.56	27.89	1.35	1.13
C.V.	0.96	0.20	0.12	0.25	0.30	0.22	0.28	0.30	0.91
Median	1.6	-6.2	41.0	2.2	0.12	21.3	846.3	37.7	8.3
Minimum	-1.6	-8.4	27.0	0.9	0.06	15.9	417.8	19.2	0.2
Maximum	4.7	-3.5	44.4	3.2	0.24	36.7	1576.4	77.2	47.2
ANOVA statistics for differences between species									
Between Group MS (df)	11.1 (2)	165.8 (2)	502.5 (2)	2.9 (2)	0.0 (2)	60.9 (2)	553539.6 (2)	1337.4 (2)	783.2 (2)
Within Group MS (df)	2.1 (143)	2.6 (143)	10.5 (278)	.15 (278)	0.0 (278)	16.2 (278)	107213.3 (278)	169.4 (278)	125.3 (278)
F-ratio	5.2	65.0	48.1	19.0	0.2	3.7	5.2	7.9	6.3
P-value	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.855	**<0.05**	**<0.01**	**<0.01**	**<0.01**

Table 2: Correlations (non-parametric Spearman's ρ) among elemental content, stable isotopic ratios, and water depth for all three species. Correlation coefficients are designated above the diagonal, P values for the pairwise comparisons are below the diagonal. Significant ($P < 0.05$) correlations are indicated in bold.

Thalassia testudinum	Depth	$\delta^{15}N$	$\delta^{13}C$	C:N	C:P	N:P	L.I.
Depth		0.113	**-0.655**	**0.377**	-0.131	**-0.322**	-0.208
$\delta^{15}N$	0.326		0.162	**0.272**	-0.073	-0.182	-0.012
$\delta^{13}C$	**<0.001**	0.158		-0.050	**0.327**	**0.400**	**0.407**
C:N	**0.001**	**0.016**	0.665		**0.281**	-0.159	0.057
C:P	0.255	0.526	**0.003**	**0.013**		**0.871**	**0.624**
N:P	**0.004**	0.110	**<0.001**	0.163	**<0.001**		**0.700**
L.I.	0.067	0.919	**<0.001**	0.621	**<0.001**	**<0.001**	

Halodule wrightii	Depth	$\delta^{15}N$	$\delta^{13}C$	C:N	C:P	N:P	L.I.
Depth		**0.415**	**-0.609**	-0.090	**-0.391**	**-0.466**	**-0.547**
$\delta^{15}N$	**0.020**		-0.210	**0.494**	-0.052	**-0.407**	**-0.423**
$\delta^{13}C$	**<0.001**	0.258		**0.364**	**0.631**	**0.559**	**0.627**
C:N	0.632	**0.005**	**0.044**		**0.613**	0.173	0.163
C:P	**0.029**	0.781	**<0.001**	**<0.001**		**0.861**	**0.809**
N:P	**0.008**	**0.023**	**0.001**	0.353	**<0.001**		**0.958**
L.I.	**0.001**	**0.018**	**<0.001**	0.381	**<0.001**	**<0.001**	

Syringodium filiforme	Depth	$\delta^{15}N$	$\delta^{13}C$	C:N	C:P	N:P	L.I.
Depth		**0.650**	**-0.477**	0.321	-0.211	**-0.459**	**-0.500**
$\delta^{15}N$	**<0.001**		**-0.407**	**0.631**	0.041	**-0.390**	**-0.412**
$\delta^{13}C$	**0.003**	**0.012**		0.019	0.253	0.279	**0.360**
C:N	**0.052**	**<0.001**	0.909		**0.377**	-0.299	-0.203
C:P	0.210	0.812	0.131	**0.022**		**0.733**	**0.668**
N:P	**0.004**	**0.017**	0.095	0.072	**<0.001**		**0.885**
L.I.	**0.002**	**0.011**	**0.029**	0.228	**<0.001**	**<0.001**	

Table 3: Parameter estimates for non-linear regressions of pairwise seagrass $\delta^{13}C$ seasonal data from 1999-2000 at 30 permanent monitoring stations in Florida Keys National Marine Sanctuary.

Species Comparison	equation	r^2	Parameter estimates (95% confidence interval) mean	amp	Φ
T.testudinum	Y = -8.94 + .539 sin(DOY radians + 3.71)	0.55	(-9.41 , -8.48)	(-.140 , 1.22)	(2.55 , 4.88)
H. wrightii	Y = -11.02 + .833 sin(DOY radians + 4.28)	0.56	(-11.79 , -10.24)	(-.208 , 1.87)	(2.92 , 5.64)
H. wrightii	Y= -10.90 +.689 sin(DOY radians + 4.19)	0.55	(-11.55 , -10.25)	(-.193 , 1.57)	(2.83 , 5.55)
S. filiforme	Y= -6.67 + .834 sin(DOY radians + 4.28)	0.55	(-7.45 , -5.88)	(-.224 , 1.89)	(2.9 , 5.66)
T. testudinum	Y= -8.96 +.433 sin(DOY radians + 3.59)	0.73	(-9.2 , -8.71)	(.068 , .797)	(2.84 , 4.35)
S. filiforme	Y= -6.99 +.605 sin(DOY radians + 4.73)	0.51	(-7.62 , -6.36)	(-.216 , 1.43)	(3.16 , 6.31)

FIGURE CAPTIONS

Figure 1. Map of study area showing locations of both survey sites and permanent monitoring sites. Survey sites have been designated as either single- or multi-species seagrass beds.

Figure 2. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Frequency distributions of elemental ratios analyzed during 1999 and 2000 across FKNMS.

Figure 3. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Frequency distributions of Limitation Index values analyzed during 1999 and 2000 across FKNMS.

Figure 4. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Frequency distributions of stable carbon isotopes analyzed during 1999 across FKNMS.

Figure 5. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Frequency distributions of stable nitrogen isotopes analyzed during 1999 across FKNMS.

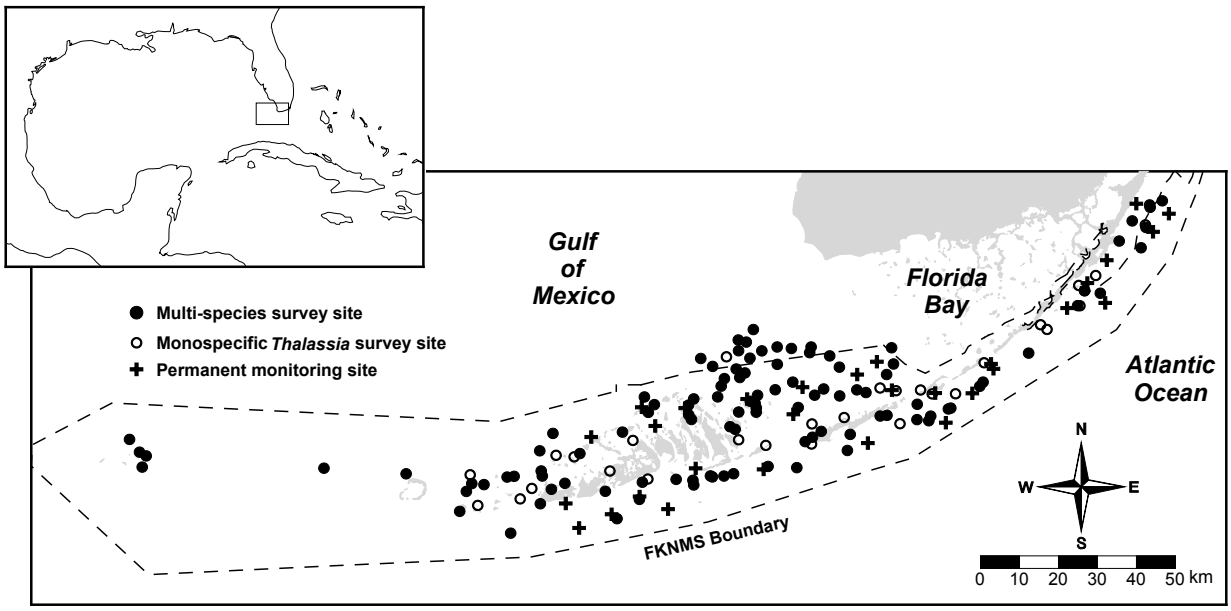
Figure 6. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Relationship between the $\delta^{13}C$ and $\delta^{15}N$ isotope ratios and depth. Linear regression and 95% confidence interval of the regression are indicated.

Figure 7. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Interspecific comparisons in N:P ratios and Limitation Index values where species co-occur. Error bars are ± 1 SE. Significant differences between species are indicated (paired T-tests, ** = $p < 0.01$). The numbers of sites at which the species pairs co-occurred are given over each pair of bars.

Figure 8. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Interspecific comparisons in $\delta^{13}C$ and $\delta^{15}N$ isotope ratios where species co-occur. Error bars are ± 1 SE. Significant differences between species are indicated (paired T-tests, ** = $p < 0.01$). The numbers of sites at which the species pairs co-occurred are given over each pair of bars.

Figure 9. *Thalassia testudinum, Halodule wrightii,* and *Syringodium filiforme*. Pairwise interspecific comparisons of seasonal patterns in $\delta^{13}C$ of green leaves at the 30 permanent monitoring stations for co-occurring species. Each point represents the mean of the sites where both species of the pairwise comparisons co-occurred. Error bars represent ± 1 SE. The best fit sine model of the form $y = \text{mean} + \text{amp} \times \sin(\text{time} + \Phi)$ is shown, where amp is the amplitude of a sine wave and Φ is a phase angle in radians (2π radians = 365 d).

FIGURE 1.


Gulf
of
Mexico
Florida
Bay
Atlantic
Ocean
Multi-species survey site
Monospecific Thalassia survey site
Permanent monitoring site
FKNMS Boundary
N
W
E
S
0 10 20 30 40 50 km

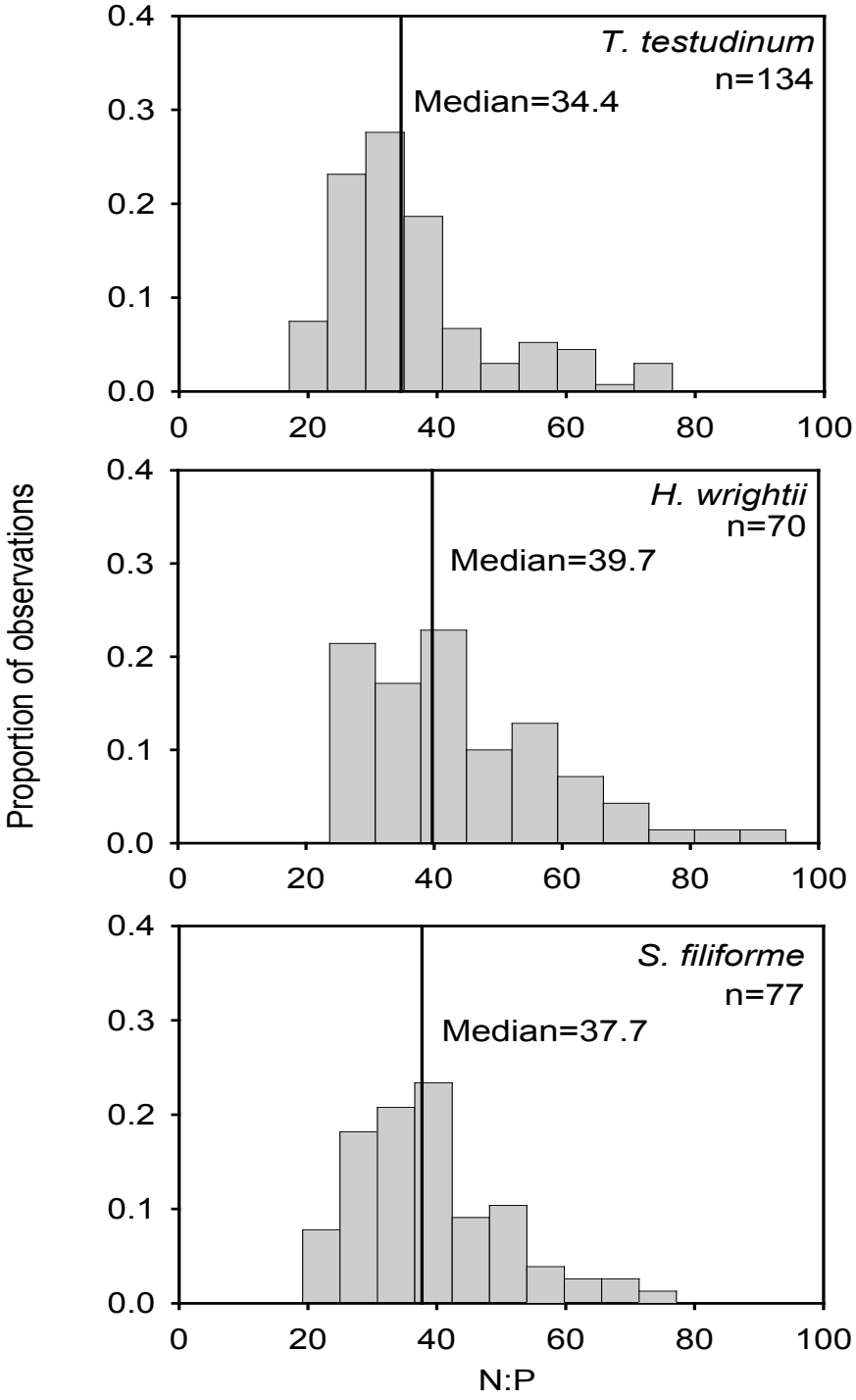

T. testudinum
n=134
Median=34.4
H. wrightii
n=70
Median=39.7
S. filiforme
n=77
Median=37.7
Proportion of observations
N:P
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
100

FIGURE 2.

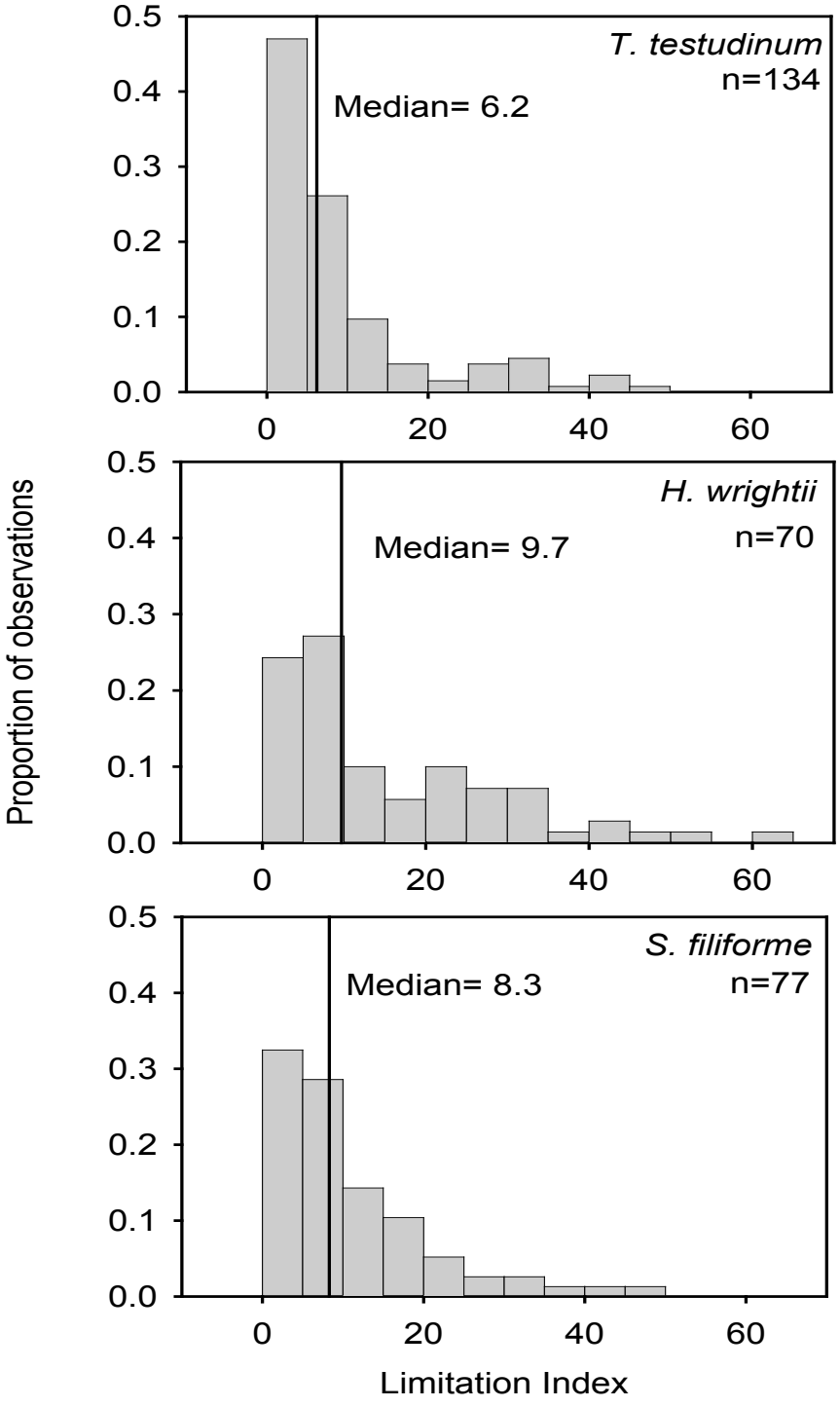

T. testudinum
n=134
Median= 6.2
H. wrightii
n=70
Median= 9.7
S. filiforme
n=77
Median= 8.3
Proportion of observations
Limitation Index
0.0
0.1
0.2
0.3
0.4
0.5
0
20
40
60

FIGURE 3.

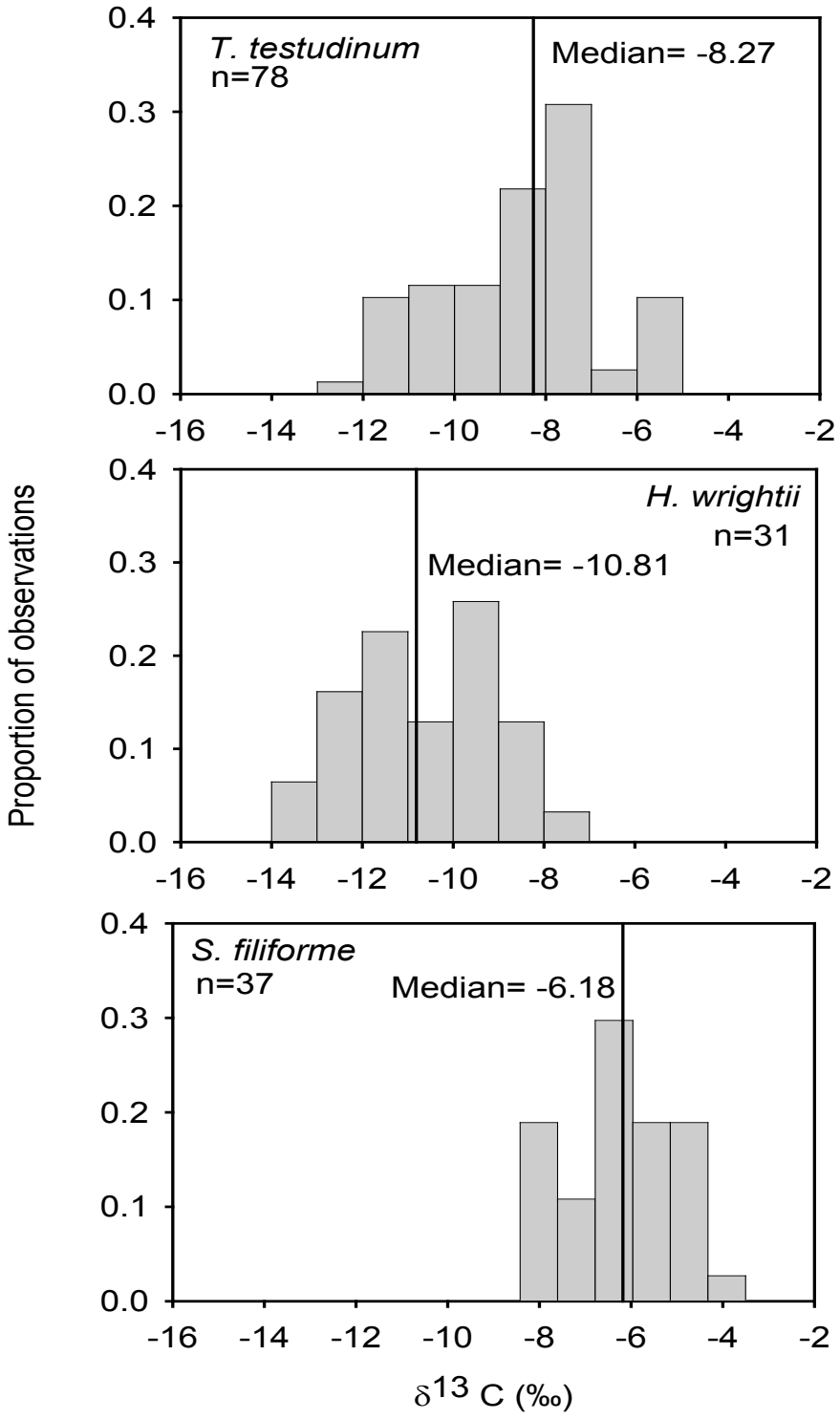

T. testudinum
n=78
Median= -8.27
H. wrightii
n=31
Median= -10.81
S. filiforme
n=37
Median= -6.18
Proportion of observations
δ13 C (‰)
0.4
0.3
0.2
0.1
0.0
-16
-14
-12
-10
-8
-6
-4
-2

FIGURE 4.

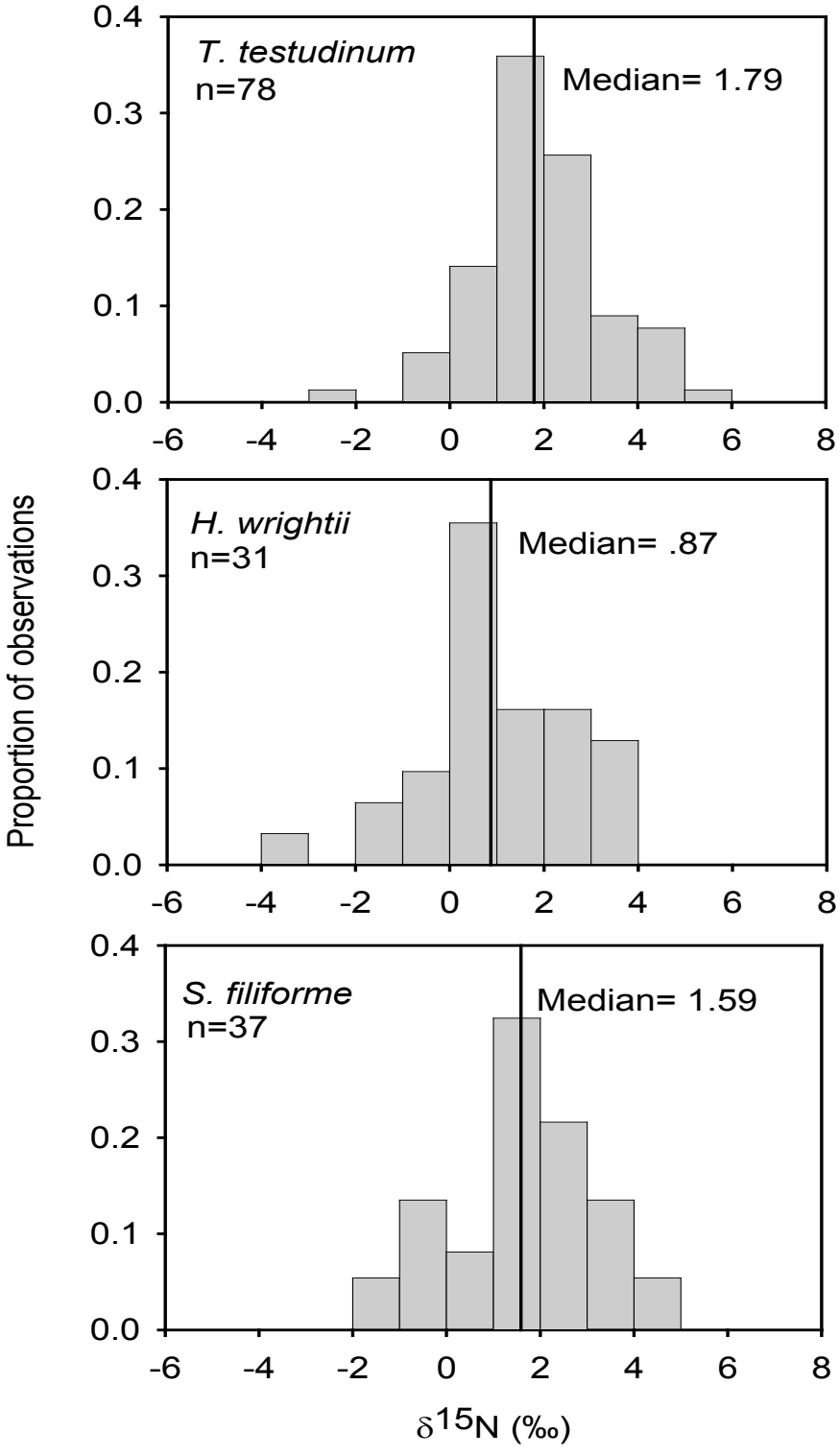

T. testudinum
n=78
Median= 1.79
H. wrightii
n=31
Median= .87
S. filiforme
n=37
Median= 1.59
Proportion of observations
δ15N (‰)
0.0
0.1
0.2
0.3
0.4
-6
-4
-2
0
2
4
6
8

FIGURE 5

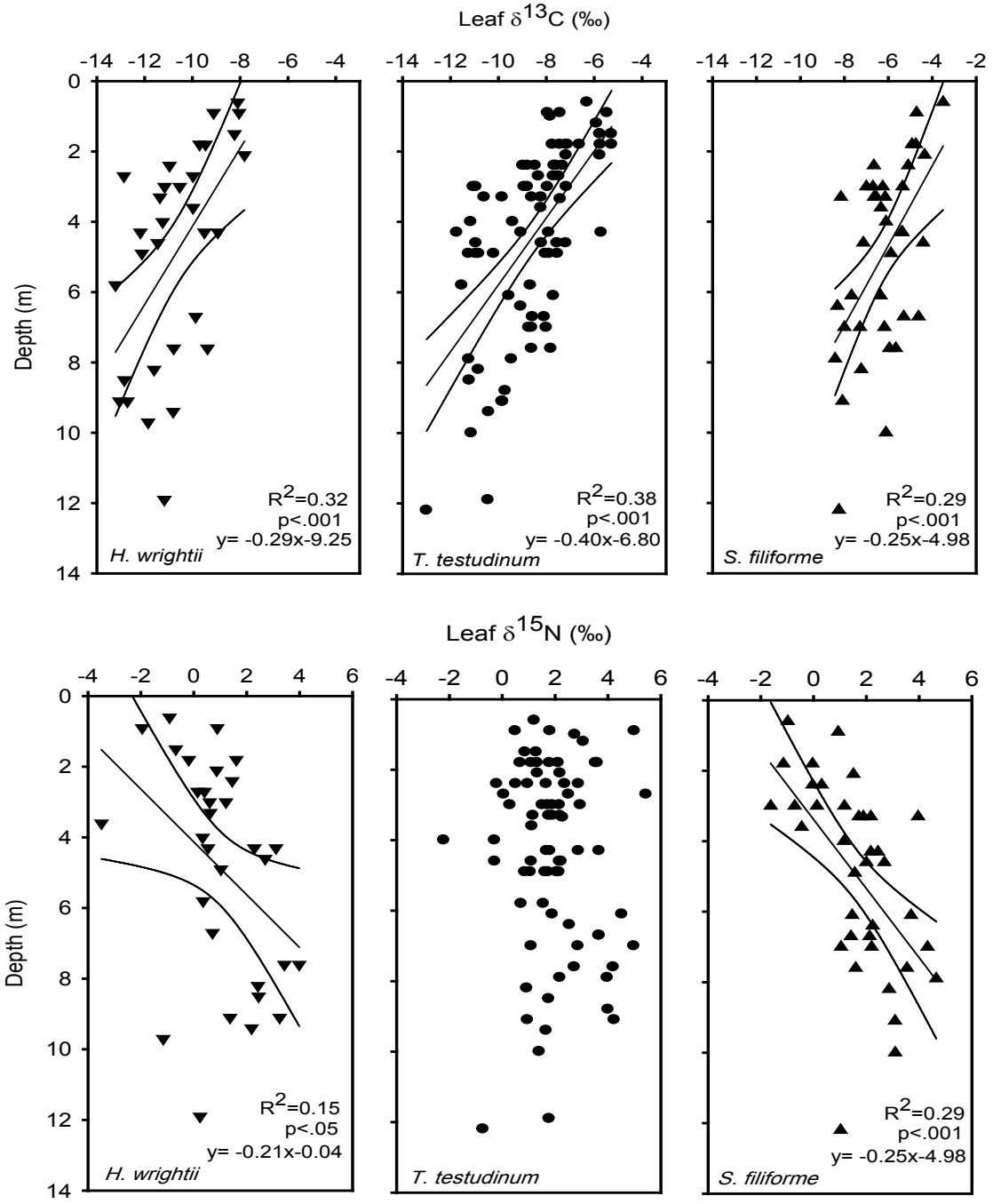

Leaf δ13C (‰)
-14 -12 -10 -8 -6 -4
-14 -12 -10 -8 -6 -4
-14 -12 -10 -8 -6 -4 -2
Depth (m)
0 2 4 6 8 10 12 14
R2=0.32
p<.001
y= -0.29x-9.25
H. wrightii
R2=0.38
p<.001
y= -0.40x-6.80
T. testudinum
R2=0.29
p<.001
y= -0.25x-4.98
S. filiforme
Leaf δ15N (‰)
-4 -2 0 2 4 6
-4 -2 0 2 4 6
-4 -2 0 2 4 6
Depth (m)
0 2 4 6 8 10 12 14
R2=0.15
p<.05
y= -0.21x-0.04
H. wrightii
T. testudinum
R2=0.29
p<.001
y= -0.25x-4.98
S. filiforme

FIGURE 6.

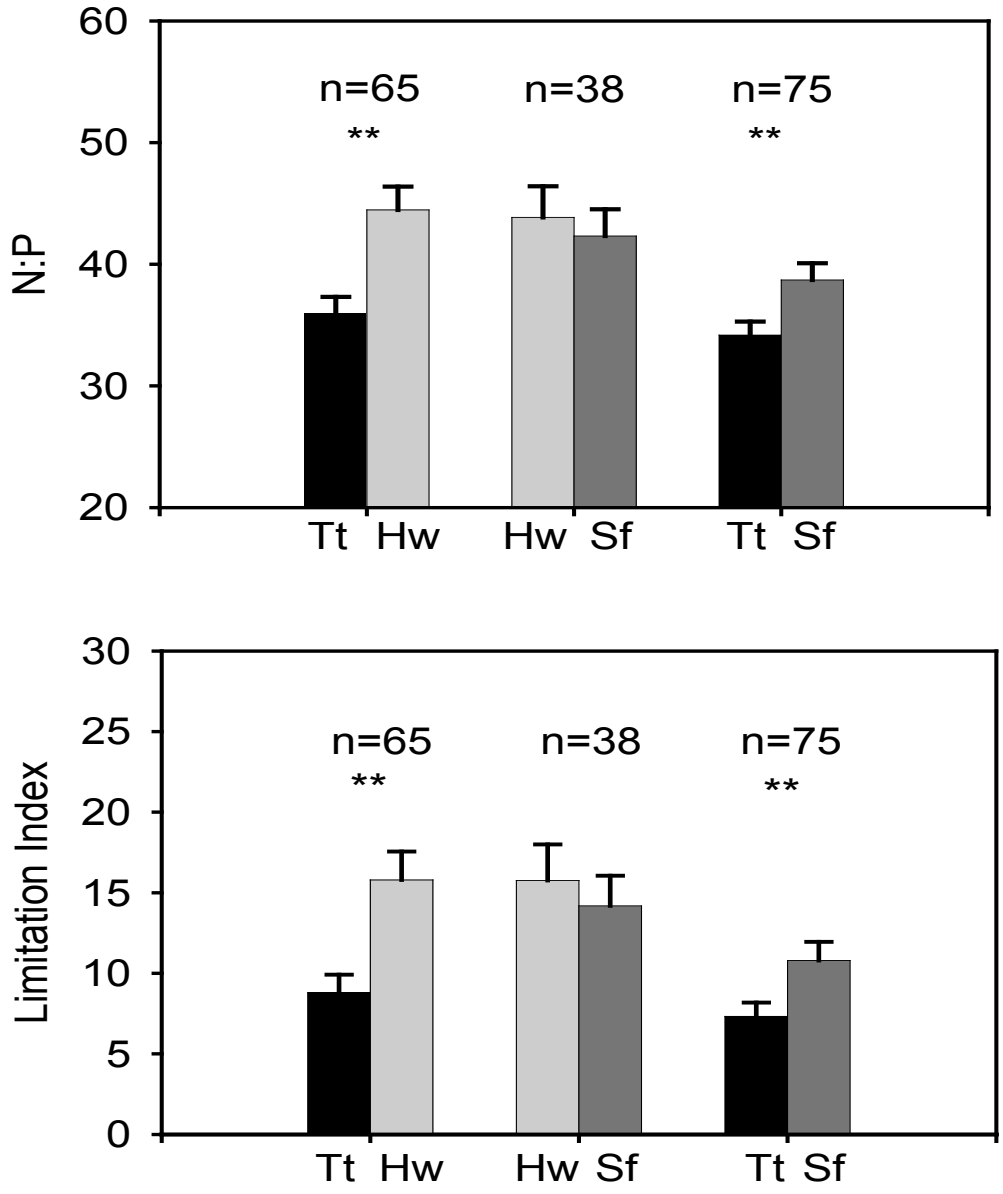

60
50
40
30
20
N:P
n=65
n=38
n=75
**
**
Tt Hw
Hw Sf
Tt Sf
30
25
20
15
10
5
0
Limitation Index
n=65
n=38
n=75
**
**
Tt Hw
Hw Sf
Tt Sf

FIGURE 7.

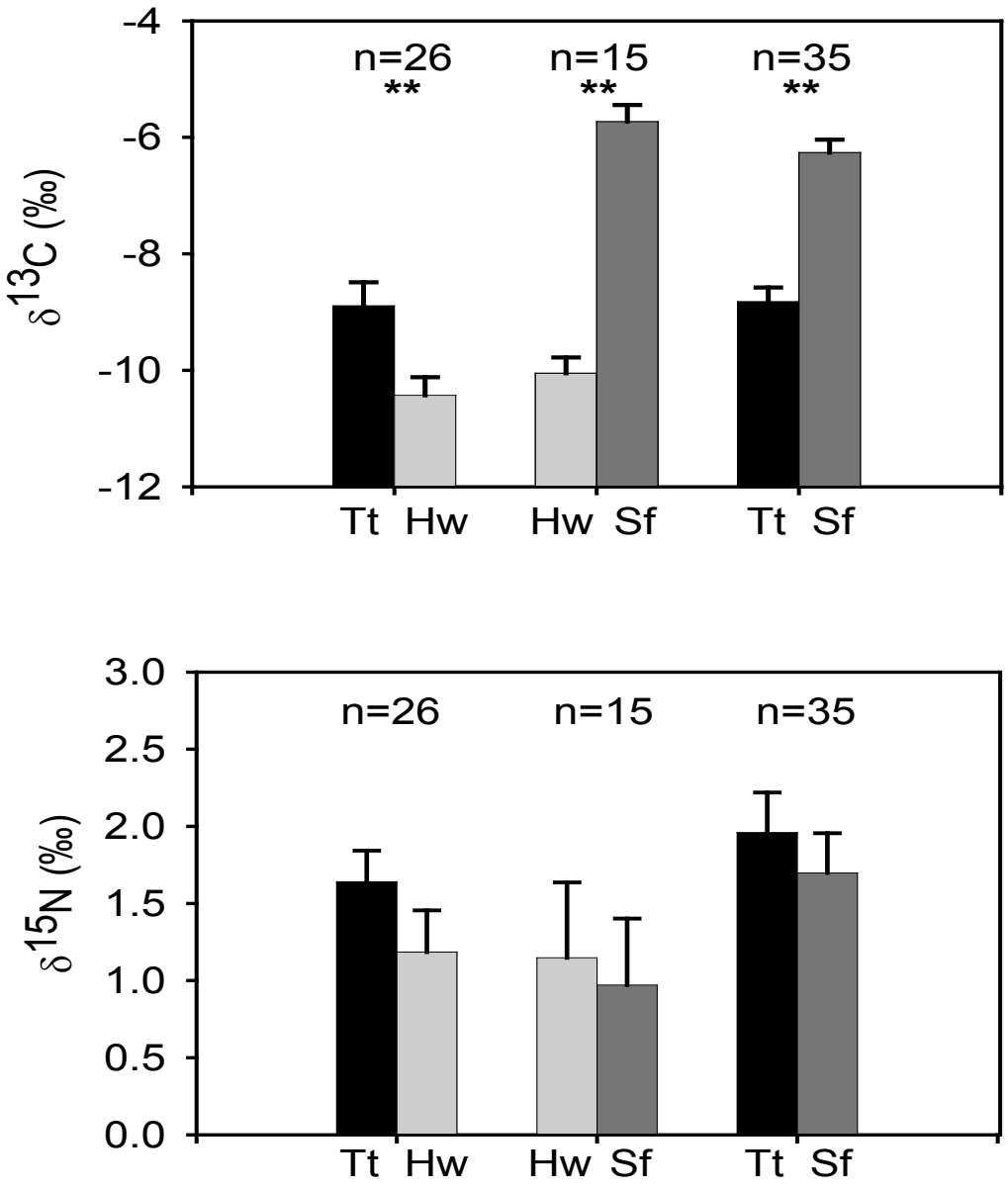

-4
-6
-8
-10
-12
δ13C (‰)
n=26
n=15
n=35
**
**
**
Tt Hw
Hw Sf
Tt Sf
3.0
2.5
2.0
1.5
1.0
0.5
0.0
δ15N (‰)
n=26
n=15
n=35
Tt Hw
Hw Sf
Tt Sf

FIGURE 8.

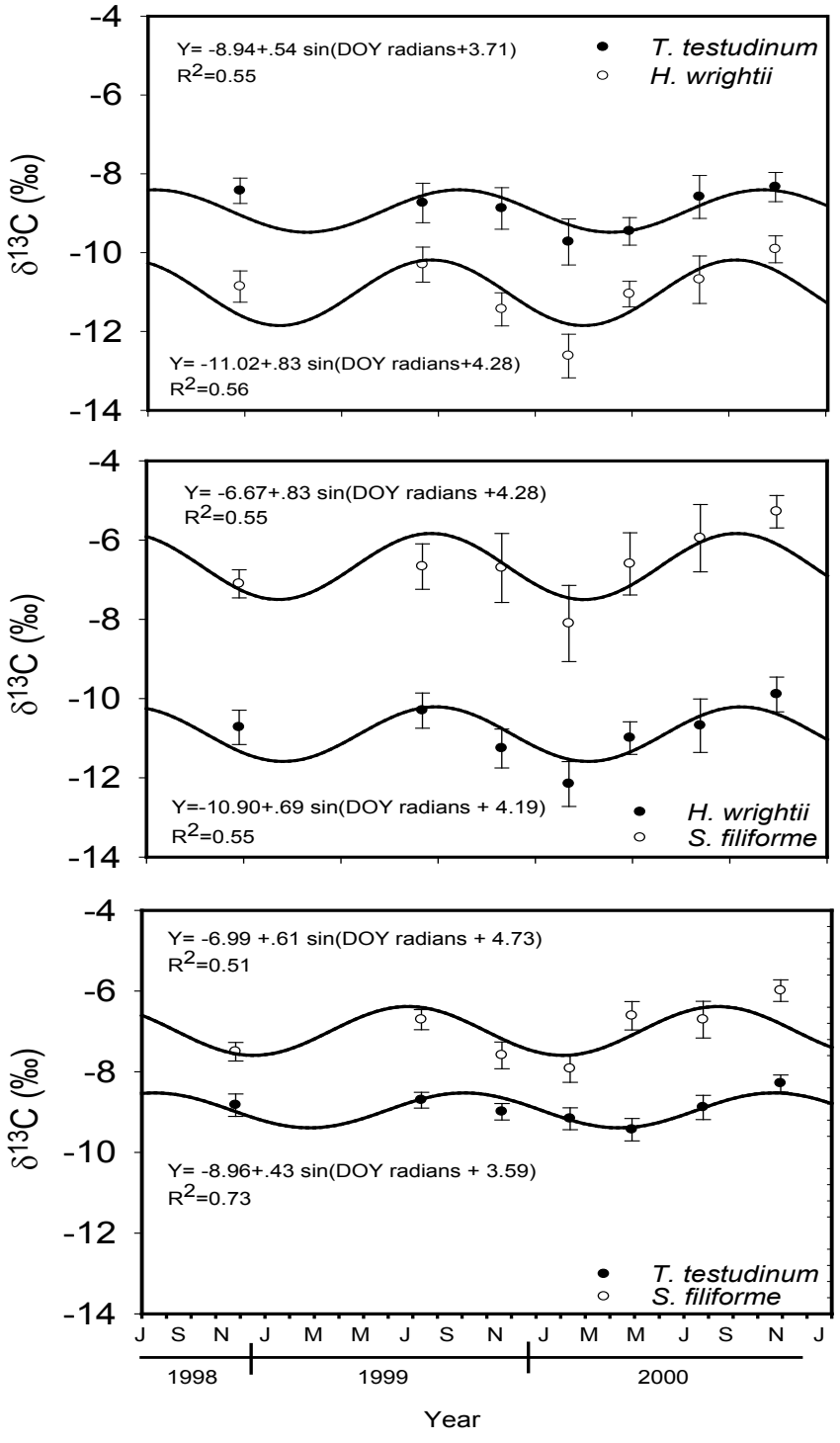

Y= -8.94+.54 sin(DOY radians+3.71)
R2=0.55
T. testudinum
H. wrightii
Y= -11.02+.83 sin(DOY radians+4.28)
R2=0.56
Y= -6.67+.83 sin(DOY radians +4.28)
R2=0.55
Y=-10.90+.69 sin(DOY radians + 4.19)
R2=0.55
H. wrightii
S. filiforme
Y= -6.99 +.61 sin(DOY radians + 4.73)
R2=0.51
Y= -8.96+.43 sin(DOY radians + 3.59)
R2=0.73
T. testudinum
S. filiforme
δ13C (‰)
-4
-6
-8
-10
-12
-14
J S N J M M J S N J M M J S N J
1998
1999
2000
Year

FIGURE 9.

CHAPTER III

NOVEL METHODOLOGY FOR IN SITU CARBON DIOXIDE ENRICHMENT OF BENTHIC ECOSYSTEMS

Campbell JE, Fourqurean JW (2011) Novel methodology for in situ carbon dioxide enrichment of benthic ecosystems. Limnology and Oceanography-Methods 9:97-109

ABSTRACT

Future climate change will likely represent a major stress to shallow aquatic and coastal marine communities around the world. Most climate change research, particularly in regards to increased pCO_2 and ocean acidification, relies on *ex situ* mesocosm experimentation, isolating target organisms from their environment. Such mesocosms allow for greater experimental control of some variables, but can often cause unrealistic changes in a variety of environmental factors, leading to "bottle effects." Here we present an *in situ* technique of altering dissolved pCO_2 within nearshore benthic communities (e.g., macrophytes, algae, and/or corals) using submerged clear, open-top chambers. Our technique utilizes a flow through design which replicates natural water flow conditions, and minimizes caging effects. The clear, open-top design additionally ensures that adequate light reaches the benthic community. Our results show that CO_2 concentrations and pH can be successfully manipulated for long durations within the open-top chambers, continuously replicating forecasts for the year 2100. Enriched chambers displayed an average 0.46 unit reduction in pH as compared to ambient chambers over a 6 month period. Additionally, CO_2 and HCO_3^- concentrations were all significantly higher within the enriched chambers. We discuss the advantages and disadvantages of this technique in comparison to other ex situ mesocosm designs utilized for climate change research.

INTRODUCTION

Since the Industrial Revolution of the early 1800s, widespread fossil fuel combustion has contributed large quantities of carbon dioxide to both atmospheric and oceanic reservoirs around the globe. Present day atmospheric CO_2 concentrations of 396 ppm represent a near 30% increase over preindustrial values, with concentrations forecast to surpass 700 ppm by the end of the century (Meehl et al. 2007). Roughly 30% of this anthropogenically released CO_2 has been absorbed by the global oceans (Sabine et al. 2004) with important consequences for the carbonate chemistry of the surface waters (Feely et al. 2004). The carbonate equilibria of marine waters can be described by a series of dissociation reactions, whereby increases in dissolved CO_2 concentrations elevate the abundance of certain carbonate species (CO_2 and HCO_3^-), while decreasing the abundance of others (CO_3^{2-}):

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \Leftrightarrow 2H^+ + CO_3^{2-}$$

Furthermore, CO_2-mediated increases in the abundance of H^+ ions are expected to dramatically reduce oceanic pH, with forecasts of a 0.3 - 0.5 unit reduction by the year 2100 (Caldeira and Wickett 2003; Raven et al. 2005). These alterations in the pH and the carbonate equilibrium of the oceanic surface waters may have substantial implications for a variety of important biotic and ecosystem processes (e.g., photosynthesis and calcification), particularly when integrated over large spatial scales. Quantifying the responses of marine ecosystems to future climate change scenarios remains an important, albeit difficult task, as some biological responses can display considerable variation (Kroeker et al. 2010).Thus, calls have been made for additional empirical research which

examines such impacts across a variety of taxonomic groups under realistic environmental conditions (Doney et al. 2009; Hendriks et al. 2010).

Much of climate change research within marine systems has been directed towards understanding how long term and pervasive changes in oceanic pCO_2 can serve as a stressor on both benthic and water column organisms, and the processes they regulate. For example, it is expected that increases in dissolved CO_2 concentrations are likely to reduce long term calcification rates across many coral reef ecosystems (Kleypas et al. 1999; Langdon et al. 2000). A variety of calcifying marine organisms; microalgae (Riebesell et al. 2000; Zondervan et al. 2001), macroalgae (Jokiel et al. 2008; Kuffner et al. 2008; Gao and Zheng 2010), and corals/invertebrates (Langdon et al. 2000; Langdon and Atkinson 2005; Shirayama and Thornton 2005; Gazeau et al. 2007; Lombard et al. 2010) have all displayed declines in $CaCO_3$ production with experimentally elevated pCO_2. Additional studies have highlighted interactive effects between temperature and pCO_2 on the calcification rates of marine organisms (Martin and Gattuso 2009; Rodolfo-Metalpa et al. 2010). Such studies clearly demonstrate implications for the resilience of coral reefs under increasing anthropogenic and climatic pressures. However, a majority of these experiments have been conducted within artificial indoor mesocosms, which can isolate target organisms from realistic natural conditions, and can fail to account for environmental variation (Hall-Spencer et al. 2008; Kleypas and Yates 2009; Hendriks et al. 2010). While some studies have begun utilizing outdoor mesocosm facilities (Riebesell et al. 2007; Jokiel et al. 2008), and field manipulations along natural pH gradients (Cigliano et al. 2010; Dias et al. 2010), we know relatively little in regards to *in situ* responses of organisms to altered pCO_2 and pH conditions.

Several studies have additionally suggested that altered pCO_2within coastal environments may have the ability to influence the functioning of aquatic and marine plant communities (Zimmerman et al. 1997; Short and Neckles 1999; Palacios and Zimmerman 2007; Hall-Spencer et al. 2008; Martin et al. 2008; Kleypas and Yates 2009). External increases in CO_2 and HCO_3^- concentrations have the ability to increase the diffusive flux of dissolved inorganic carbon (DIC) across leaf boundary layers, and have been shown to increase seagrass production (Hall-Spencer et al. 2008), leaf photosynthetic rates (Durako 1993; Beer and Koch 1996; Invers et al. 1997; Zimmerman et al. 1997), and plant reproductive output (Palacios and Zimmerman 2007). Other studies have additionally demonstrated changes to the seagrass epiphyte community under various CO_2 enrichment scenarios, with large declines in biogenic carbonate production, and potential biogeochemical shifts within these shallow, coastal systems (Martin et al. 2008). Submerged macrophytes comprise much of the coastal benthic community around globe and are important contributors to the carbon sink capacity of the world's oceans (Duarte et al. 2011). Thus, similar to declines in coral reef calcification, changes in oceanic pCO_2 may additionally have widespread implications for these productive and economically important ecosystems.

Experiments which address CO_2-mediated responses of benthic plants have likewise mainly been restricted to mesocosm designs (Titus et al. 1990; Titus 1992; Zimmerman et al. 1997; Pagano and Titus 2007; Palacios and Zimmerman 2007). Aquarium and mesocosm facilities generally provide optimal conditions to encourage vigorous plant and/or algal growth, thus responses detected within the laboratory may not hold true for natural communities, where alternate resources may become increasingly

limiting under elevated CO_2 loads. Several terrestrial studies have documented disparities between *ex situ* and *in situ* responses in regards to the impacts of CO_2 enrichment on plant community dynamics (Ainsworth and Long 2005). Within these systems, CO_2 mediated growth responses can be rapidly constrained by the availability of other essential resources, such as water and/or nutrients (Diaz et al. 1993).

Many mesocosm pCO_2 experiments control seawater carbonate equilibrium via either acid addition (which shifts the relative concentrations of the carbonate species with no increase in total DIC) or CO_2 bubbling (which simultaneously increases the abundance of CO_2, HCO_3^- and total DIC). While it has been suggested that the latter technique of CO_2 enrichment best replicates forecasted changes in seawater carbonate parameters (Hurd et al. 2009; Schulz et al. 2009), few studies have attempted to transition this *ex situ* methodology towards an *in situ* design (Barry et al. 2010). Here we describe a novel technique of long term CO_2 enrichment applicable to the study of changes in pCO_2 on the productivity and functioning of aquatic and marine benthic communities. Our methodology consists of a submerged array of clear, open-top, flow-through acrylic chambers, which allows for continuous CO_2 enrichment over long time periods. Our system utilizes an efficient technique of *in situ* CO_2 bubbling, which maximizes the dissolution and containment of CO_2 gas, while minimizing losses to the external water column and atmosphere. Furthermore, the open, flow-through design allows for ample light to reach target organisms (macrophytes, corals, and/or algae), while reducing caging effects. Easy access to the benthic community through the open tops of the enclosures additionally allows for accurate measurements of carbonate parameters (pH and alkalinity), and a variety of biotic processes (plant/algal growth rates, calcification rates,

and photosynthetic fluorometric responses). This system provides an inexpensive and efficient technique of in situ pCO_2 manipulation around a wide variety of benthic communities to study various climate change/ocean acidification scenarios.

METHODS

Site description

In situ pCO_2 manipulation was initiated on 1 July 2009 within a shallow, nearshore benthic plant community within the Florida Keys, Florida, USA (24.55° N, 81.75° W). The benthic community was dominated by the seagrass *Thalassia testudinum*, with lower abundances of the seagrasses *Syringodium filiforme* and *Halodule wrightii*. A variety of calcareous green algal species (*Halimeda* spp. and *Penicillus* spp.) were additionally present within the seagrass canopy, along with a substantial epiphyte community (dominated by filamentous turf and coralline encrusting algae) growing on the seagrass leaf surfaces. The sediments were composed of roughly 10% organic matter; with the remaining mineral fraction consisting of mud-sized biogenic calcium carbonates, and larger particles of the carbonate skeletal remains of a variety of calcifying organisms. The experimental seagrass bed was located 10m offshore, within a shallow (1m depth) embayment, which exchanged waters with the Gulf of Mexico to the northwest, and had an average salinity of 37. Noon light levels below the water surface averaged 1000 (µmol photons m^{-2} s^{-1}), while light levels at the top of the seagrass canopy averaged 800 (µmol photons m^{-2} s^{-1}) for the duration of the experiment. CO_2 enrichment was conducted continuously for a period of 6 months until 1 January 2010.

Experimental design and chamber description

The benthic CO_2 enrichment experiment consisted of three replicated (n=5) treatments, arranged in a complete randomized block design: 1) CO_2 enrichment within clear, open- top chambers; 2) no CO_2 enrichment within clear, open-top chambers; and 3) control plots lacking clear open top chambers. Treatments were arranged within a grid design (3 rows x 5 columns), with each column representing a separate complete block (Fig. 1). Treatments were randomly assigned within each block. This design was necessary to organize the plumbing system utilized to deliver CO_2-rich seawater to the enriched chambers. Each chamber was constructed of 4 optically-clear, acrylic panels (0.8m x 0.4m x 0.4m), producing a total chamber volume of $0.13m^3$ and enclosing a benthic area of $0.17m^2$. Each chamber was assembled utilizing corrosion resistant (zinc plated) 3.8 cm corner braces, with adjacent acrylic panels additionally sealed at the corners with an adhesive. Each chamber was anchored to the benthos utilizing a series of plywood panels and cinder blocks. Each chamber received 4 plywood panels (.4m x .1m) that were affixed to the base of each acrylic side utilizing cable ties. Cinder blocks were then placed on top of each panel to provide a tight seal between the bottom of the chamber and the substrate (Fig. 2). Short, 2.5 cm diameter PVC segments were hammered into the sediment, down to the bedrock, underneath each plywood panel to provide support for each cinder block, and prevent compaction of the surrounding sediment. This system allowed the chambers accommodate turbulent wave energy experienced in the field, while remaining firmly fixed in place. Chambers were cleaned of fouling organisms on a bi-monthly basis, and light measurements both within and

outside several replicate chambers were conducted periodically with a submersible 2pi light sensor (WALZ Diving PAM).

CO_2 enrichment system

In situ carbon enrichment was achieved by rapidly mixing gaseous CO_2 with ambient seawater to achieve the desired pH. The rate of gas delivery was set to reproduce dissolved CO_2 concentration forecasts for the year 2100 (Caldeira and Wickett 2003) within our enriched chambers. These forecasts were selected as target values because they represent "business-as-usual" scenarios, whereby CO_2 emissions continue along current trends (Raven et al. 2005). To achieve enrichment, a 34 kg cylinder of compressed CO_2 (AirGas, beverage grade) was connected to a two-stage regulator with a fine control valve, which bled gas into a submerged water pump (120 V AC, 6800LPH, 145W). Once bled into the intake port of the pump, the CO_2 was rapidly mixed with ambient seawater by the impeller, and then delivered to the carbon enriched chambers via a PVC plumbing network. Gas flow rates were incrementally increased until the pH within the enriched chambers was reduced by the target value of 0.4 units in comparison to the controls. Once established, CO_2 flow to the pumps remained constant, resulting in a constant, reduced pH within the enriched chambers compared to the surrounding environment. Gas pressure from the two-stage regulator was set at 15 psi, while the gas flow rate was set at 1.12 L/min. Flexible, CO_2-proof tubing (Cramer Decker, 150 meters) was used to deliver CO_2 from the cylinder to the submerged pump. A second water pump was established without connection to a CO_2 line and delivered ambient seawater to the unenriched chambers via a secondary independent PVC plumbing network. Both

submerged powerheads were caged within a mesh PVC frame (1m x 1m x 0.5m) to exclude debris. Industrial garden hose (2 cm diameter, 10m in length) was connected to the outflow of each water pump and utilized to deliver CO_2-enriched and ambient seawater to the PVC plumbing network.

PVC plumbing network

Seawater from each respective water pump was redirected to a series of independent submerged PVC pipes (one enriched line, one ambient line) that divided the main seawater flow amongst the replicates for each chamber treatment. Each garden hose from the powerheads was connected to a PVC pipe (3.8 cm diameter, 10m in length) that ran lengthwise along the southern border of the chamber array (Fig. 1). Each PVC pipe contained 5 separate 3 cm hose bibbs which were evenly spaced at 1 m intervals (corresponding to the spacing between the grid columns). Industrial garden hose (2 cm diameter) was then utilized to deliver seawater from the hose bibb to each clear acrylic chamber, through a 1.9 cm diameter hole drilled through the base of the chamber. Each hose bibb along the PVC pipe could then be finely adjusted to equalize seawater flow between the chambers, which was measured at 340 liters/hour. Thus, the water volume within each chamber was replaced approximately 63 times daily.

Measurement of seawater carbonate parameters

Seawater carbonate parameters within each chamber were monitored periodically throughout the 6 month experiment. Temperature and pH inside the seagrass canopy within each chamber and control plot were recorded 53 separate times, across 18 distinct sampling dates. Six of the sampling dates represent diurnal measurements, when pH and

temperature were recorded every 3 hours from 900 to 2100. This sampling schedule produced over 800 pH measurements, allowing us to describe how carbonate parameters varied amongst treatments both throughout the day, and during the enrichment period. The pH was recorded using a handheld Thermo Scientific Orion 4-Star meter, calibrated with NBS standards (relative accuracy ±0.002). Temperature was additionally recorded once every hour during the 6 month period by an underwater Onset Temp Logger, one within a randomly selected chamber, and a second within a randomly selected control plot. On a monthly basis, replicate diurnal (morning and evening) water samples (20ml) were collected within each chamber and control plot, filtered through a 0.7 μm GFF filters, and stored on ice within borosilicate glass containers until further processing. Upon return to the lab, total alkalinity was measured by automated, potentiometric titration with 0.1 N HCL. Salinity was recorded with an Orion conductivity meter. Carbonate parameters ($CO_{2(aq)}$, HCO_3^-, CO_3^{2-} , and calcite/aragonite saturation states) were calculated with the CO_2Sys Excel Macro (Lewis and Wallace 1998), using the dissociation constants of Mehrbach et al. (1973), refit by Dickson and Millero (1987).

Stable Isotope Measurements

Stable carbon isotope ($\delta^{13}C$) measurements were additionally used to further demonstrate the effectiveness of our CO_2 enrichment technique. If our carbon enriched treatments were effective in increasing the diffusive flux of CO_2 to the benthic seagrass community, we hypothesized significant reductions in the $\delta^{13}C$ signature of the seagrass tissue growing within the enriched chambers, in accordance with previous correlative and experimental studies (Durako and Sackett 1993; Vizzini et al. 2010). Every month,

aboveground leaf material from 3-5 shoots of *T. testudinum* was collected within each chamber and control plot, scraped free of adhered epiphytes, separated according to leaf age, dried in the lab for 48 hrs at 60^{o}C, and ground to a fine power. Isotope analyses were conducted on the youngest leaf material (leaf rank 1) utilizing standard elemental analyzer isotope ratio mass spectrometer procedures. An elemental analyzer was used to combust all organic material and subsequently reduce the formed gasses into N_2 and CO_2, which were measured on a Finnigan MAT Delta C IRMS in a continuous flow mode. The samples' isotopic ratios (R) are reported in the standard delta notation (δ):

$$\delta(‰) = [(R_{sample}/R_{standard})\text{-}1] \times 1000$$

These results are presented with respect to the international standards of atmospheric nitrogen (N_2) and Vienna Pee Dee belemnite (V-PDB) for carbon. Analytical reproducibility of the reported δ values, based on sample replicates, was better than ± 0.08‰ for carbon. Samples of the CO_2 source gas used for enrichment were additionally collected for stable isotopic analysis. Airtight, double-ended Swagelock sample cylinders were utilized to sample10 of the Airgas CO_2 cylinders during the enrichment period. The stable carbon isotopic composition of this source CO_2 was determined by directly injecting each gas sample into a dual inlet ratio mass spectrometer calibrated against a reference gas of a known isotopic value.

Statistical analysis

Seawater carbonate parameters were analyzed by comparing the 95% confidence intervals of the mean water quality measurements within the chambers and control plots throughout the enrichment period (Table 1). As a result of diurnal variation, water quality

measurements were compared within 5 distinct time segments (900-1200; 1200-1500; 1500-1800; 1800-2100; 2100-0000). Monthly measurements of seagrass stable carbon isotopes within each of the treatments were analyzed using a repeated-measures analysis of variance (ANOVA, $\alpha = 0.05$), with month as the within-subject factor, and CO_2 treatment as the between-subject factor. When significance was detected, a Bonferroni corrected post-hoc analysis was performed**.**

RESULTS

For the duration of the six month field enrichment period, our open-chamber, flow -through system functioned continuously without failure. The carbonate parameters within the enriched chambers were responsive to CO_2 addition from the gas cylinder, and displayed significantly reduced pH, and higher $CO_{2(aq)}$ and HCO_3^- concentrations throughout the enrichment period as compared to the unenriched chambers and control plots (Fig. 3-6; Table 1). Conversely, CO_3^{2-} concentrations, calcite saturation, and aragonite saturation states were all significantly reduced within the enriched chambers (Fig. 5,6; Table 1). There were no statistical differences in carbonate parameters between the unenriched chambers and control plots during any of the diurnal time segments.

Background carbonate parameters did vary throughout the day (reflecting water column and benthic processes of photosynthesis and respiration), however, the enriched chambers remained significantly enriched during each time segment throughout the 6 month period (Fig. 6). Temperature additionally varied both diurnally, and throughout the enrichment period, however during all sampling dates, there were no statistical differences between treatments. Total alkalinity ranged from an average of 2200 μmol kg^-

^{1} in July, to an average of 2569 μmol kg^{-1} in November, agreeing with other water quality measurements for Western regions of Florida Bay (Millero et al. 2001). Within each monthly sampling, total alkalinity was not influenced by CO_2 enrichment, nor were significant differences detected between the morning and afternoon alkalinity measurements, suggesting that the dissolution of carbonate sediments was not increased by CO_2 addition. Salinity displayed slight variation during the enrichment period, with values of 37.1 in July to 36.9 in November. Light measurements revealed that the chamber design did impose a slight reduction in light reaching the macrophyte canopy. Such reductions were highest (≈5%), in the early morning and late afternoon when much of the irradiance was penetrating the water column at an oblique angle, and transmitted through the chamber panel. Light reductions were minimal during the noon hours when irradiance passed perpendicular to the water surface, and through the open top of the chamber. Overall, light reductions were lowest during the critical noon period when photosynthetic rates for benthic communities are generally at their highest (Yates et al. 2007).

Water quality measurements throughout the 6 month period reveal that our chamber design was effective in, 1) reaching our target enrichment range forecasted for the year 2100, and 2) continuously holding those values within that range over long temporal periods. Median pH values within the CO_2 enriched chambers, CO_2 ambient chambers, and control plots were 7.75, 8.21, and 8.21 respectively (Fig. 3). On average, enriched chambers displayed a 0.46 unit reduction in pH values, as compared to the other unenriched treatments (Fig. 4).

For 87% of the pH sampling dates, the enriched chambers were held within ± 0.2 pH units of our target value, with chambers being held to within ± 0.1 pH units for 61% of the sampling dates.

Stable carbon isotope measurements demonstrate that the benthic seagrass community growing within the enriched chambers did experience an increase in carbon flux to the photosynthetic tissues for the duration of the experiment (Fig. 7). Such measurements integrate information about the local carbon environment over long time periods and provide strong biological evidence of CO_2 enrichment. In accordance with our hypotheses, seagrass stable carbon isotope values were significantly reduced within the CO_2 enriched chambers as compared to the unenriched treatments for all sampling periods during the experiment ($F = 63.74$, $p < 0.01$). There were no statistical differences in seagrass $\delta^{13}C$ values between the unenriched chambers and the control plots. The within-subjects factor of time was significant during the experiment ($F = 7.03$, $p<0.01$), strongly driven by the increasingly negative $\delta^{13}C$ values within the enriched treatments over the course of the experiment. The interaction between time and treatment was additionally significant ($F = 12.66$, $p<0.01$) because there was only a change in the $\delta^{13}C$ of seagrass tissues from the CO_2-enriched chambers. The stable carbon isotope composition of the source CO_2 gas used for enrichment displayed slight variation, with a median value of -4.32‰.

DISCUSSION

In the current study, we explore the potential utility of open-top chambers (OTCs) as a means of *in situ* carbon enrichment within shallow aquatic or marine benthic

environments. We submit that a number of factors must be met in order to make long-term carbon enrichment via this technique feasible; 1) significant increases in carbon supply must be detected within the CO_2 enriched chambers over long time periods, demonstrating the ability of this design to deliver gaseous CO_2 from an onshore location to a submerged benthic location, 2) unnecessary confounding effects such as light and/or water flow reductions must be minimized within all chambers, and 3) the OTC design must sufficiently constrain CO_2 parameters such that target enrichment values are achieved for the majority of the enrichment period.

Our carbonate parameter measurements show that the carbonate environment within the CO_2 enriched chambers was dramatically altered in comparison to the CO_2 ambient chambers and the control plots. The open top design allowed for sufficient containment of injected CO_2, and produced an acceptable rate of gas consumption for the size of the chamber array and our target enrichment level (approx. 68 kg of CO_2/month). A larger array, with greater replication, could easily be implemented using this design, via the addition of extra chambers, or a complete duplication of the entire array. Overall, the possible level of carbon enrichment using this technique will depend upon the trade-off between chamber number, chamber size, and desired enrichment level. We submit that some pilot testing may be necessary to pinpoint the appropriate number of gas cylinders required, and the gas delivery rate from the CO_2 regulator to the submerged water pumps.

The arrangement of the PVC enrichment system additionally allows for careful adjustment of the water flow rates both within and between chamber treatments. Water flow rates can be individually adjusted to compensate for the loss of pressure head

between chambers adjacent to the water pumps and those which are at the opposite end of the array. Using larger pipe diameters to deliver seawater would reduce friction head within the system, and increase the efficiency of the submerged pumps. While restricted to nearshore environments, the use of larger, more powerful water pumps may alleviate such constrains, allowing for the placement of enrichment chambers at locations further offshore.

The flow-through, open-top chambers allowed for minimal caging confounds in regards to light and water flow conditions. Light reductions, on average, were no greater than 5%. Bi-monthly cleaning of both the internal and external surfaces of the chambers prevented algal fouling and maintained adequate light levels for the duration of the experiment. Light reduction within the chambers at noon was minimal, as chamber light levels often displayed similar values to the unchambered control plots. The response of benthic communities to CO_2 enrichment may strongly depend upon ambient light levels, thus the high levels displayed within our design (800 μmol photons m^{-2} s^{-1}) ensure realistic values experienced by many shallow benthic communities. The continuous operation of the water pumps ensured that the chamber design did not strongly influence other water quality variables throughout the experiment. All water quality measurements and carbonate calculations (temperature, pH, alkalinity, salinity, $CO_{2(aq)}$, HCO_3^-, and CO_3^{2-}) were similar between the unenriched chambers and the unchambered control plots, suggesting that water flow was adequate to prevent biologically mediated shifts in water quality within each chamber. Thus, our OTC design served to only manipulate carbonate parameters within our enriched chambers, while not altering other water chemistry variables.

The OTC design displayed moderate constraint of carbonate parameters within the CO_2 enriched treatments for the duration of the experiment (Fig. 4). Over the 6 month period, an average difference of 0.47 pH units was maintained between the CO_2 treatments throughout the day (Fig. 6). In comparison, mesocosm studies generally display a tighter constraint of CO_2 parameters both within enriched and unenriched treatments due to the use of pH monitors coupled to solenoid gas valve regulators. For our *in situ* design, a number of complex environmental factors contributed to the variation in pH values across chamber treatments over time, attributable to both ambient background variation (which equally impacted all chambers), and turbulent weather conditions (which only impacted the CO_2 enriched treatments). Background variation caused both diurnal and seasonal variation amongst all treatments, and demonstrates the ability of biotic processes (photosynthesis and respiration) to control local carbonate parameters when water column mixing is relatively low, as previously documented for other regions of Florida Bay (Yates and Halley 2006). Turbulent weather from periodic storm events differentially increased carbonate parameter variation within our CO_2 enriched treatments. High wind events increased the flushing rate of ambient seawater into the open-top chambers, and temporarily reduced the effectiveness of our enrichment treatment (pulling the pH and carbonate parameters towards background values). While impacting several of our sampling dates, these storm events were short-lived, and thus did not reduce the effectiveness of our CO_2 treatments over the long term. Conversely, periods of low wind energy served to increase the effectiveness of our CO_2 enrichment. In comparison, pH variation within our enriched treatments was of similar magnitude to other *in situ* observations that have examined the impacts of ocean acidification on

benthic communities near volcanic vents, which naturally lower pH and increase $CO_{2(aq)}$ concentrations (Hall-Spencer et al. 2008; Martin et al. 2008). The overall precision of the control of carbonate parameters within the enriched treatments could be largely improved by incorporating a pH dependent feedback loop, similar to many mesocosm studies. Use of a variable-flow, magnetic solenoid valve (regulated by a pH monitor), would control gas delivery into the submerged water pumps and automatically regulate gas flow according to external wind conditions, providing greater constraint of carbonate parameters within the enriched treatments.

Stable carbon isotope measurements of the macrophyte community within the chambers and control plots suggests that over long time periods, vegetation within the enriched treatments was exposed to significantly elevated $CO_{2(aq)}$ concentrations as compared to the unenriched treatments. The increasingly negative seagrass $\delta^{13}C$ values with CO_2 enrichment agree with our original hypotheses, and possibly reflect an effect of concentration-dependent isotope fractionation (Fig.7). The $\delta^{13}C$ value of plant material can be influenced by both the isotopic composition of the source DIC pool, and photosynthetic carbon isotope discrimination (Farquhar et al. 1989). Increases in the external supply of DIC impact plant $\delta^{13}C$ values by allowing for increased discrimination against the heavier carbon isotope (^{13}C) during photosynthetic carbon fixation (Smith and Walker 1980). Thus, our shifts in $\delta^{13}C$ with carbon enrichment are consistent with these statements, and are in agreement with previous studies which demonstrate that an elevated $CO_{2(aq)}$ supply results in increasingly negative $\delta^{13}C$ values within both seagrass tissues and marine macroalgae (Durako and Sackett 1993; Kubler et al. 1999; Vizzini et al. 2010).

Shifts in the isotopic composition of the source DIC can additionally contribute to changes in the isotopic composition of plant material. Therefore, the calculation of isotopic fractionation factors would be the preferred technique of demonstrating that isotopic shifts in plant material are related to changes in DIC concentration. While we did characterize the isotopic composition of the source CO_2 gas used for enrichment, we did not characterize the isotopic composition of the background DIC pool, thus could not conclusively determine if the change in $\delta^{13}C$ we observed in seagrass leaves was caused by a change in fractionation. However, the source CO_2 used for enrichment had a median $\delta^{13}C$ value of -4.32 ‰, which is relatively heavy compared to dissolved atmospheric CO_2 (-9.0 ‰) (Kroopnick 1985). Thus, despite adding CO_2 which displayed a relatively enriched $\delta^{13}C$ carbon signal, we produced seagrass leaf tissue with progressively depleted $\delta^{13}C$ values, suggesting an increase in the external supply of DIC. These data provide further evidence that our *in situ* chamber design was effective in achieving a long-term and persistent increase in carbon supply to our benthic community.

Throughout the 6-month period, our system was quite robust and amenable to future long-term carbon enrichment studies. While intense tropical storms did not impact South Florida during the 2009 summer season, our experimental array was subjected to a number of short-term local storms. Short periods of high wind/ wave energy showed no short- or long-term impact on the integrity or positioning of the submerged chambers. We were additionally unable to detect any significant erosion or damage to the benthic community within any of the chambers after storm events.

Lastly, we submit that our technique of *in situ* carbon enrichment is feasible in regards to both overall cost and system maintenance. Acquisition of materials and

construction/installation of the chamber array can be completed within a relatively short timeframe (2-3weeks), with an approximate budget of US$ 5000. Once established, 1-2 hours of bi-monthly maintenance is required to thoroughly clean the chambers of algal overgrowth, and clear the pumps of any collected debris. For the size of our given array and level of enrichment, the CO_2 cylinder needed to be replaced on a bi-monthly basis. The onshore location of the gas cylinder, and nearshore location of the experimental chambers facilitates the ease of the maintenance schedule, and requires a minimal number of personnel. Continued costs for CO_2 gas and electricity for the submerged pumps totaled US$ 45/month.

Our system of open-top, flow-through chambers was effective in conducting long-term *in situ* benthic CO_2 enrichment within nearshore coastal communities. Such a design answers calls for climate change research which increasingly replicates realistic field conditions, and studies the effects of altered pCO_2 over long time periods. Furthermore, this experimental design can be used to study climate change responses across multiple scales, ranging from organismal physiological responses, to ecosystem level responses such as primary production and nutrient cycling (particularly in regards to benthic plant/algal communities). Our benthic enrichment system was used to target enrichment levels forecasted for the year 2100, however alternate enrichment targets could easily be replicated by adjusting gas delivery to the submerged array. As currently designed, constraint of the carbonate parameters within the chamber array was less precise as compared to *ex situ* mesocosm studies. We suggest that higher constraint could be achieved by incorporating a pH dependent feedback, utilizing a gas solenoid linked to a pH monitor. Another weakness in this design is its relative restriction to nearshore

environments. We submit that there are a number of larger, more powerful water pumps which could be used to increase the number of potential locations accessible by this design. Larger pumps could additionally be used to increase chamber replication within the system. Overall, we feel that our *in situ* CO_2 enrichment system has potential for future aquatic and marine climate change studies, particularly those focused on addressing organismal, community, and ecological scale responses of benthic algal/plant assemblages to increased pCO_2 and ocean acidification scenarios.

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FIGURE CAPTIONS

Figure 1.Schematic of chamber array. Solid boxes designate an acrylic chamber supplied with either CO_2 enriched seawater or CO_2 ambient seawater. Dashed boxes designate control plots with no chamber. Red and blue lines represent CO_2 enriched and unenriched seawater respectively. Gray arrows indicate direction of water flow.

Figure 2. Photograph of an enrichment chamber.

Figure 3. Frequency distributions of measured pH within CO_2 enriched chambers, CO_2 ambient chambers, and control plots during the 6 month enrichment period.

Figure 4. Frequency distribution of enrichment levels during the 6 month enrichment period. Delta pH (Δ pH) represents the average difference in pH between the enriched and ambient chambers for each respective sampling date.

Figure 5. Carbonate parameters (mean ± 1 S.E.) within CO_2 enriched chambers, CO_2 ambient chambers, and control plots during the 6 month enrichment period. Values represent treatment averages observed during the 1200-1500 time period for each respective date.

Figure 6. Seasonal carbonate parameters (means ± 1 S.E.) within CO_2 enriched chambers, CO_2 ambient chambers, and control plots. Values within each time segment represent averages of 7-10 distinct sampling dates throughout the enrichment period.

Figure 7.Stable carbon isotope values (means ± 1 S.E.) of aboveground leaf material of the seagrass *Thalassia testudinum* during the enrichment period. The June sampling date represents initial seagrass stable carbon isotope values prior to CO_2 enrichment. The dashed line represents the median isotopic signature of the source CO_2 gas used for enrichment.

Table 1: Diurnal seawater carbonate parameters observed within the seagrass canopy in the CO_2 enriched chambers, CO_2 ambient chambers, and control plots during the 6 month enrichment period. Measurements are divided into 5 distinct 3-hour time groups. Carbonate parameters (means plus 95% confidence intervals) represent seasonal averages of 7-10 distinct sampling dates. Total alkalinity averaged 2345 µmol kg^{-1}, while temperature averaged 29.9°C during the enrichment period.

Time of day (observations)	pH (NBS scale)			CO_2 (µmol/kg SW)		
	CO_2 enriched	CO_2 ambient	Control Plot	CO_2 enriched	CO_2 ambient	Control Plot
0900-1200 (8)	7.57 (7.49-7.65)	8.02 (7.96-8.08)	8.02 (7.96-8.08)	62.9 (51.5-74.4)	16.8 (14.2-19.4)	16.6 (14.1-19.1)
1200-1500 (9)	7.78 (7.72-7.84)	8.23 (8.17-8.29)	8.24 (8.18-8.3)	32.2 (25.6-38.9)	8.8 (6.9-10.7)	8.7 (6.8-10.7)
1500-1800 (10)	7.74 (7.62-7.86)	8.22 (8.14-8.30)	8.22 (8.14-8.30)	42.2 (25.8-58.6)	9.4 (7.4-11.4)	9.5 (7.5-11.5)
1800-2100 (7)	7.74 (7.6-7.88)	8.30 (8.26-8.34)	8.30 (8.26-8.34)	39.0 (22.4-55.6)	6.7 (5.7-7.7)	6.7 (5.7-7.7)
2100-0000 (7)	7.62 (7.44-7.80)	8.23 (8.17-8.29)	8.24 (8.18-8.3)	59.4 (19.7-99.0)	8.2 (6.8-9.6)	8.0 (6.6-9.4)

Time of day (observations)	pCO2 (µatm)			DIC (µmol/kg SW)		
	CO_2 enriched	CO_2 ambient	Control Plot	CO_2 enriched	CO_2 ambient	Control Plot
0900-1200 (8)	2451 (1903-2999)	651 (543-759)	644 (542-746)	2291 (2184-2398)	2104 (1987-2222)	2102 (1983-2221)
1200-1500 (9)	1310 (1035-1584)	357 (281-431)	352 (278-428)	2168 (2042-2294)	1919 (1786-2051)	1917 (1783-2050)
1500-1800 (10)	1730 (1055-2405)	381 (304-457)	385 (309-461)	2235 (2123-2347)	1972 (1842-2101)	1975 (1845-2104)
1800-2100 (7)	1654 (945-2364)	282 (241-322)	282 (244-320)	2162 (2056-2268)	1845 (1752-1939)	1846 (1754-1939)
2100-0000 (7)	1982 (931-3033)	345 (287-402)	336 (279-393)	2149 (2064-2235)	1848 (1780-1916)	1842 (1773-1912)

Time of day (observations)	HCO_3^- (µmol/kg SW)			CO_3^{2-} (µmol/kg SW)		
	CO_2 enriched	CO_2 ambient	Control Plot	CO_2 enriched	CO_2 ambient	Control Plot
0900-1200 (8)	2148 (2044-2251)	1904 (1785-2023)	1900 (1779-2021)	81.2 (70.7-91.7)	183.8 (167.9-199.8)	185.4 (168.5-202.3)
1200-1500 (9)	2013 (1892-2133)	1636 (1498-1774)	1632 (1493-1771)	123.4 (110.8-136.0)	274.4 (246.5-302.3)	276.0 (248.2-303.7)
1500-1800 (10)	2072 (1973-2171)	1686 (1552-1821)	1691 (1557-1825)	121.5 (100.1-142.9)	276.9 (245.3-308.4)	274.9 (244.1-305.8)
1800-2100 (7)	2000 (1880-2120)	1526 (1416-1635)	1526 (1420-1633)	123.7 (93.4-154.0)	313.5 (295.7-331.4)	313.2 (296.7-329.7)
2100-0000 (7)	2005 (1908-2103)	1568 (1483-1652)	1559 (1472-1645)	97.5 (72.4-122.6)	272.4 (250.4-294.4)	275.8 (254.3-297.4)

Time of day (observations)	$\Omega_{Calcite}$			$\Omega_{Aragonite}$		
	CO_2 enriched	CO_2 ambient	Control Plot	CO_2 enriched	CO_2 ambient	Control Plot
0900-1200 (8)	1.9 (1.7-2.2)	4.4 (4.0-4.8)	4.4 (4.0-4.8)	1.3 (1.1-1.5)	2.9 (2.7-3.2)	3.0 (2.7-3.2)
1200-1500 (9)	3.0 (2.7-3.3)	6.6 (5.9-7.2)	6.6 (5.9-7.3)	2.0 (1.8-2.2)	4.4 (4.0-4.9)	4.4 (4.0-4.9)
1500-1800 (10)	2.9 (2.4-3.4)	6.6 (5.9-7.4)	6.6 (5.8-7.3)	2.0 (1.6-2.3)	4.5 (4.0-5.0)	4.4 (4.0-4.9)
1800-2100 (7)	3.0 (2.3-3.7)	7.5 (7.1-7.9)	7.5 (7.1-7.9)	2.0 (1.5-2.5)	5.1 (4.8-5.4)	5.1 (4.8-5.4)
2100-0000 (7)	2.3 (1.7-2.9)	6.5 (6.0-7.1)	6.6 (6.1-7.1)	1.6 (1.2-2.0)	4.4 (4.1-4.8)	4.5 (4.1-4.8)

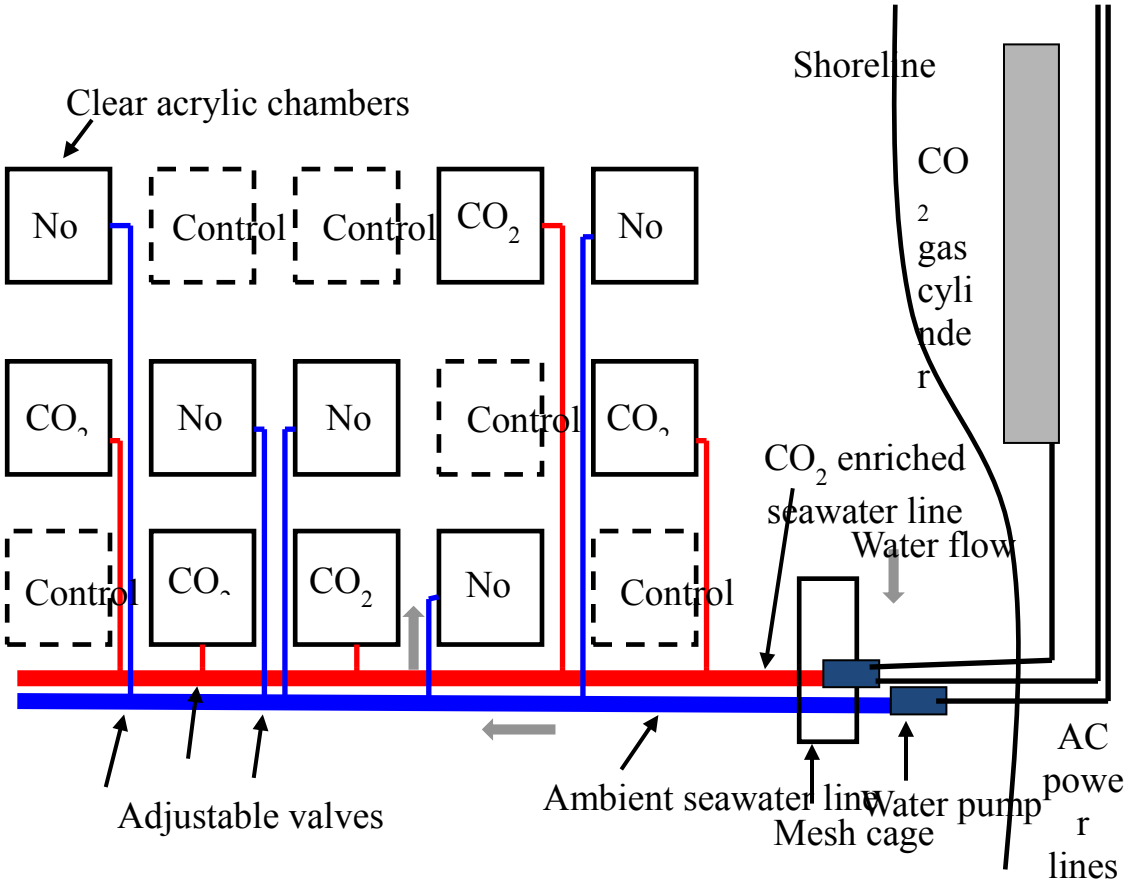

Clear acrylic chambers
Shoreline
CO2 gas cylinder
No
Control
Control
CO2
No
CO2
No
No
Control
CO2
Control
CO2
CO2
No
Control
CO2 enriched seawater line
Water flow
Adjustable valves
Ambient seawater line
Mesh cage
Water pump
AC power lines

FIGURE 1.

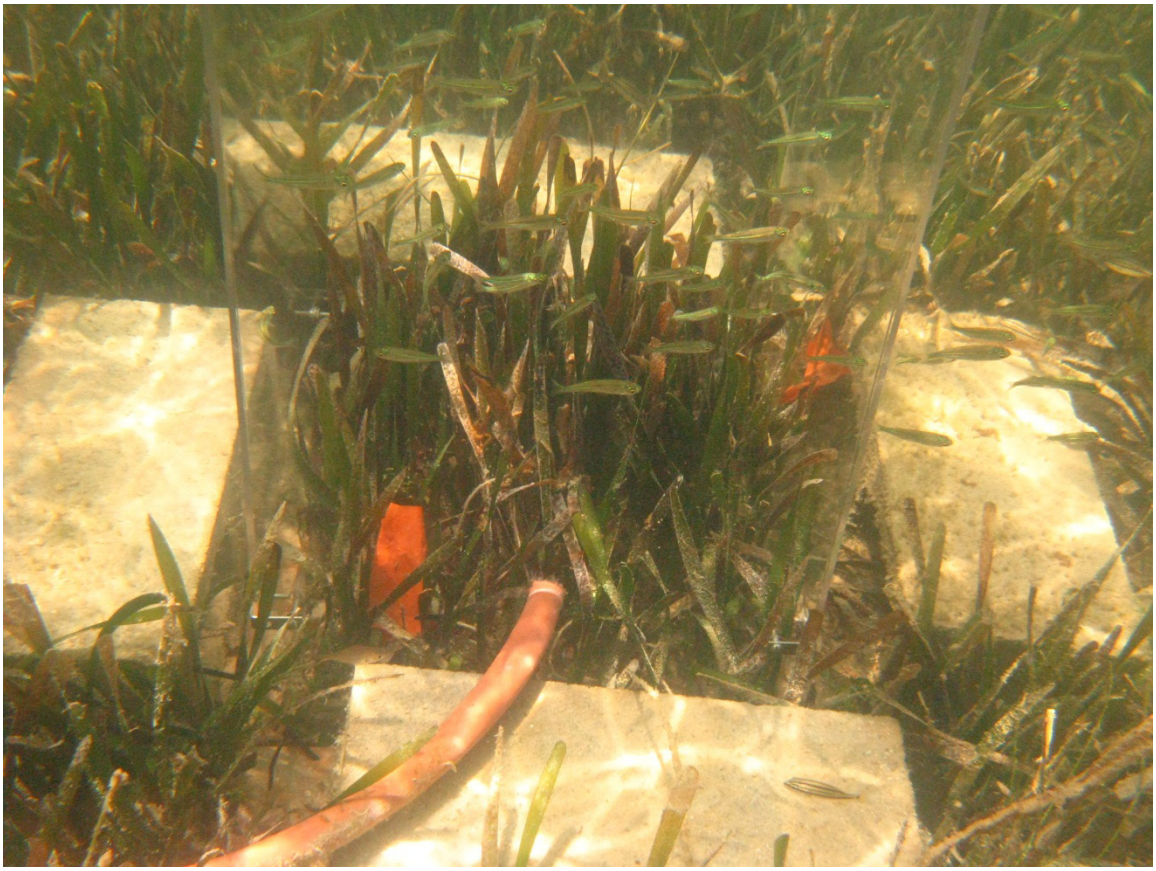


FIGURE 2.

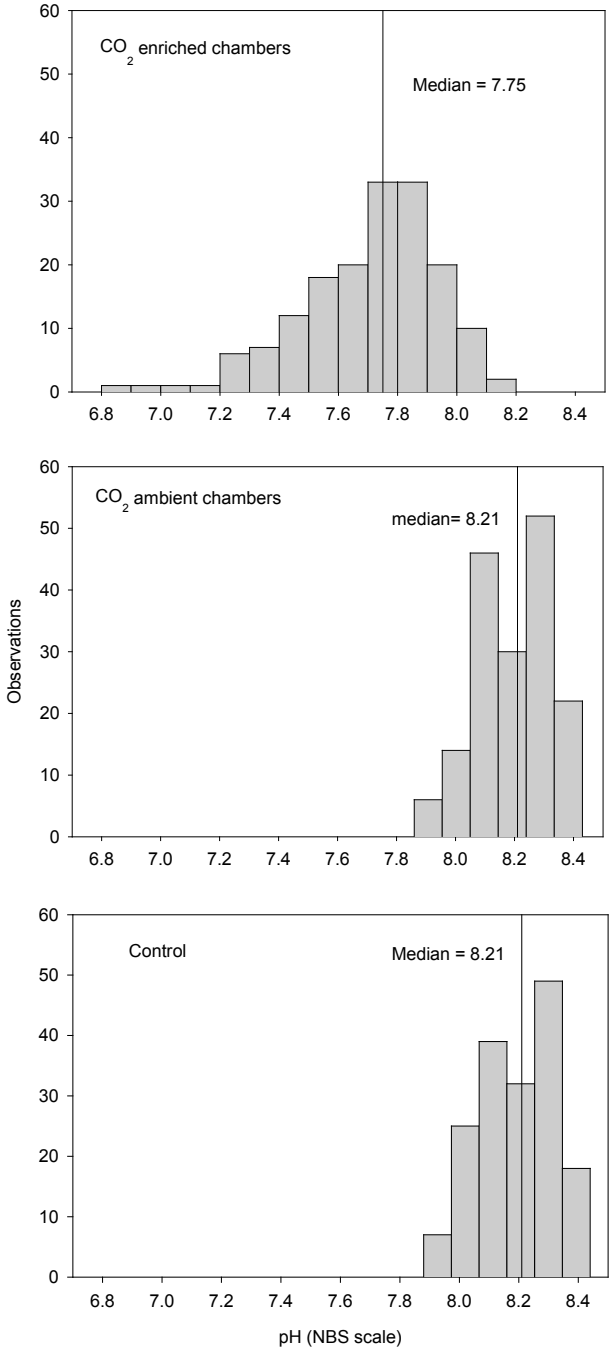

CO2 enriched chambers
Median = 7.75
CO2 ambient chambers
median= 8.21
Control
Median = 8.21
Observations
pH (NBS scale)
0
10
20
30
40
50
60
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4

FIGURE 3.

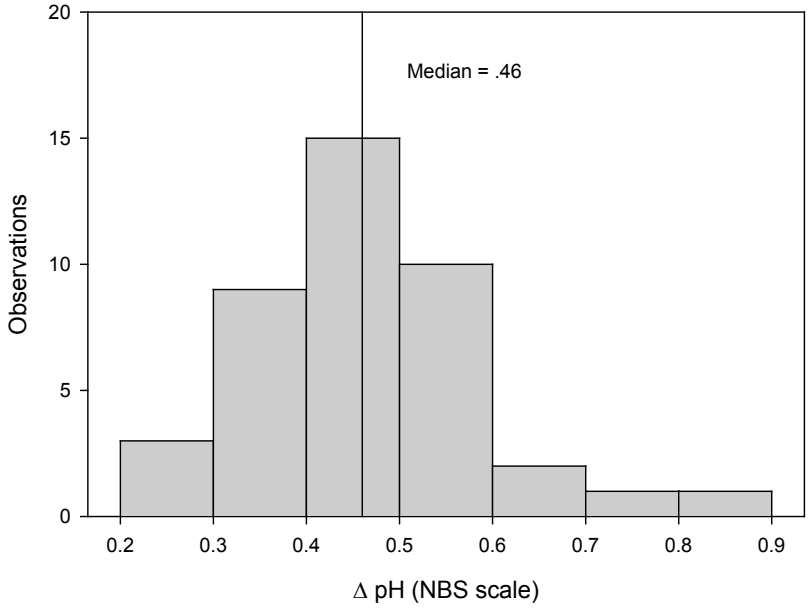
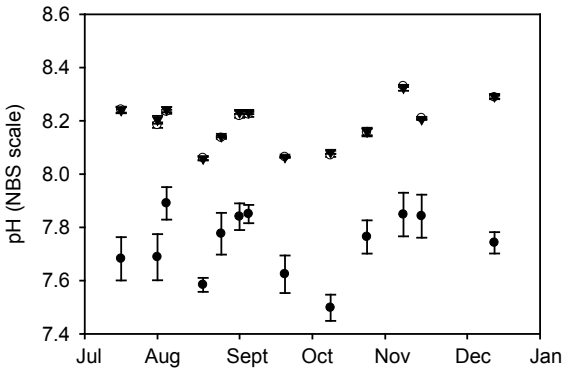
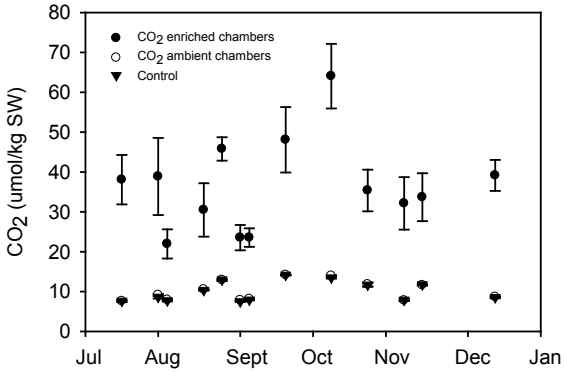
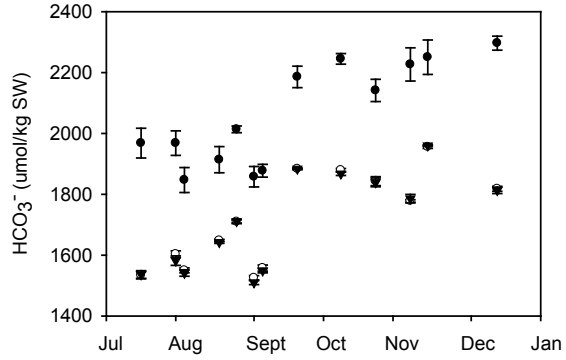
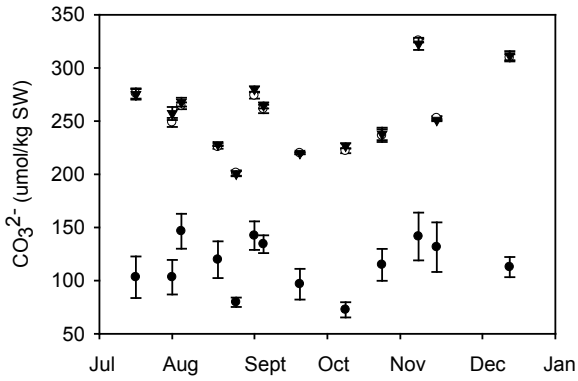


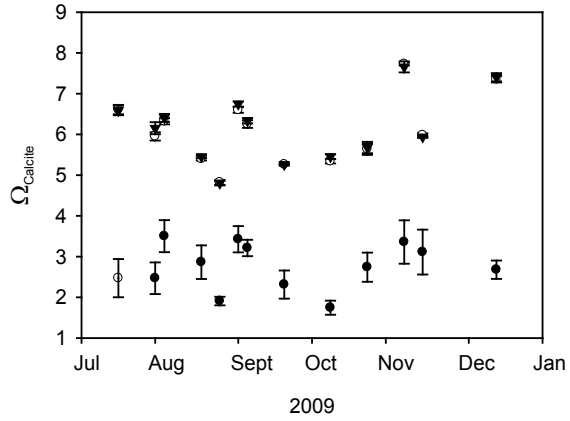
FIGURE 4.


pH (NBS scale)
8.6
8.4
8.2
8.0
7.8
7.6
7.4
Jul
Aug
Sept
Oct
Nov
Dec
Jan


CO_2 enriched chambers
CO_2 ambient chambers
Control
CO_2 (umol/kg SW)
80
70
60
50
40
30
20
10
0
Jul
Aug
Sept
Oct
Nov
Dec
Jan


HCO_3^- (umol/kg SW)
2400
2200
2000
1800
1600
1400
Jul
Aug
Sept
Oct
Nov
Dec
Jan


CO_3^{2-} (umol/kg SW)
350
300
250
200
150
100
50
Jul
Aug
Sept
Oct
Nov
Dec
Jan


$\Omega_{Calcite}$
9
8
7
6
5
4
3
2
1
Jul
Aug
Sept
Oct
Nov
Dec
Jan
2009

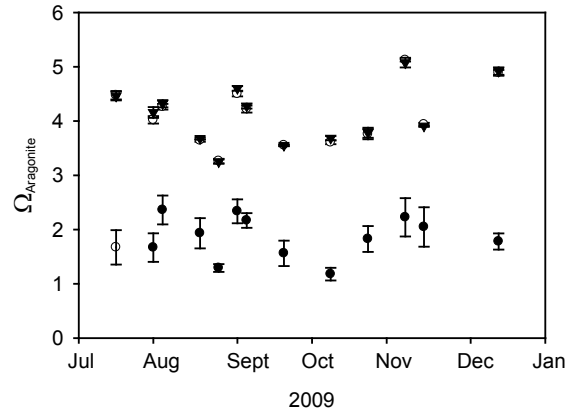

$\Omega_{Aragonite}$
6
5
4
3
2
1
0
Jul
Aug
Sept
Oct
Nov
Dec
Jan
2009

FIGURE 5.

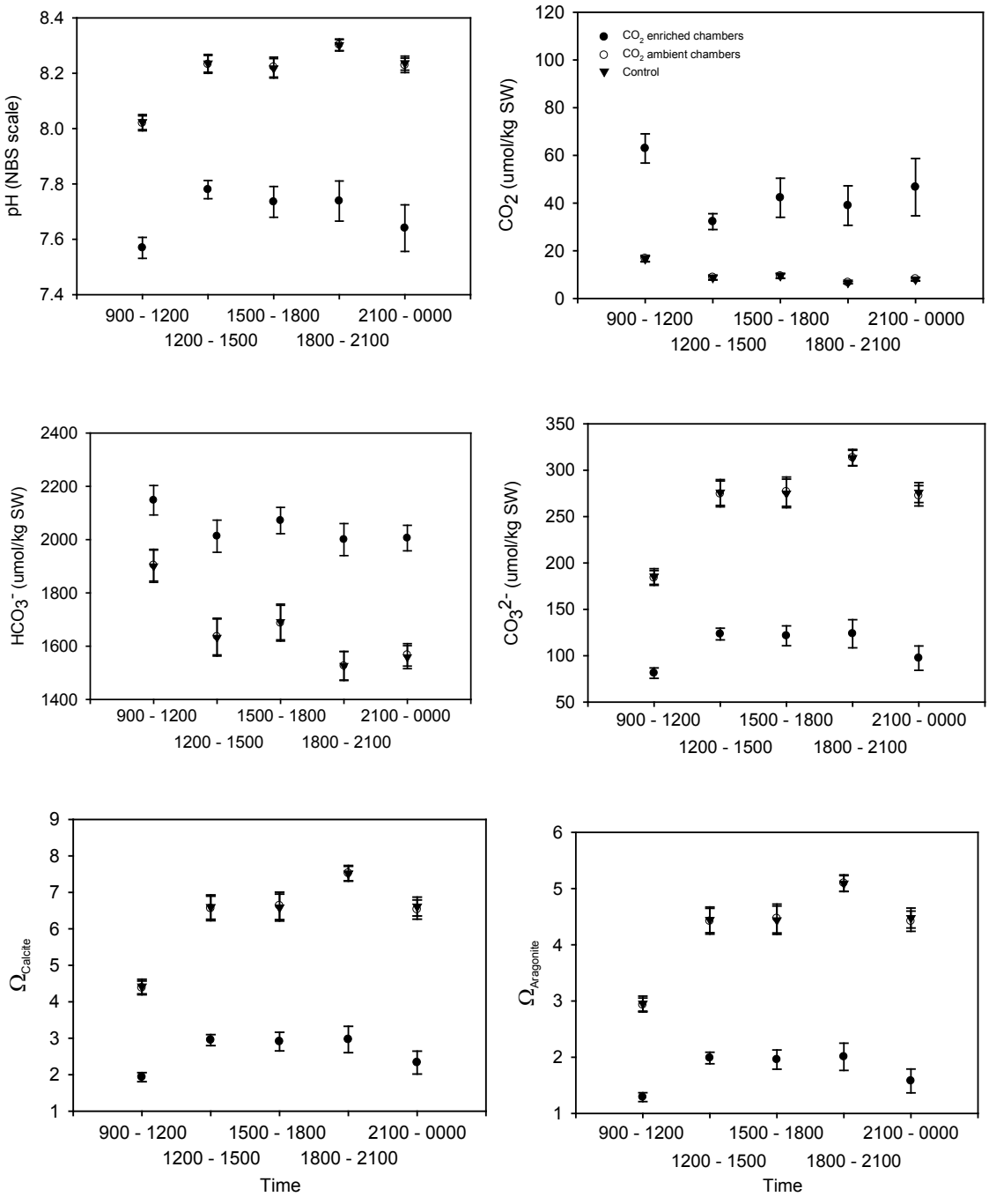

pH (NBS scale)
8.4
8.2
8.0
7.8
7.6
7.4
900 - 1200
1500 - 1800
2100 - 0000
1200 - 1500
1800 - 2100
CO2 (umol/kg SW)
120
100
80
60
40
20
0
CO2 enriched chambers
CO2 ambient chambers
Control
HCO3- (umol/kg SW)
2400
2200
2000
1800
1600
1400
CO32- (umol/kg SW)
350
300
250
200
150
100
50
ΩCalcite
9
8
7
6
5
4
3
2
1
ΩAragonite
6
5
4
3
2
1
Time

FIGURE 6.

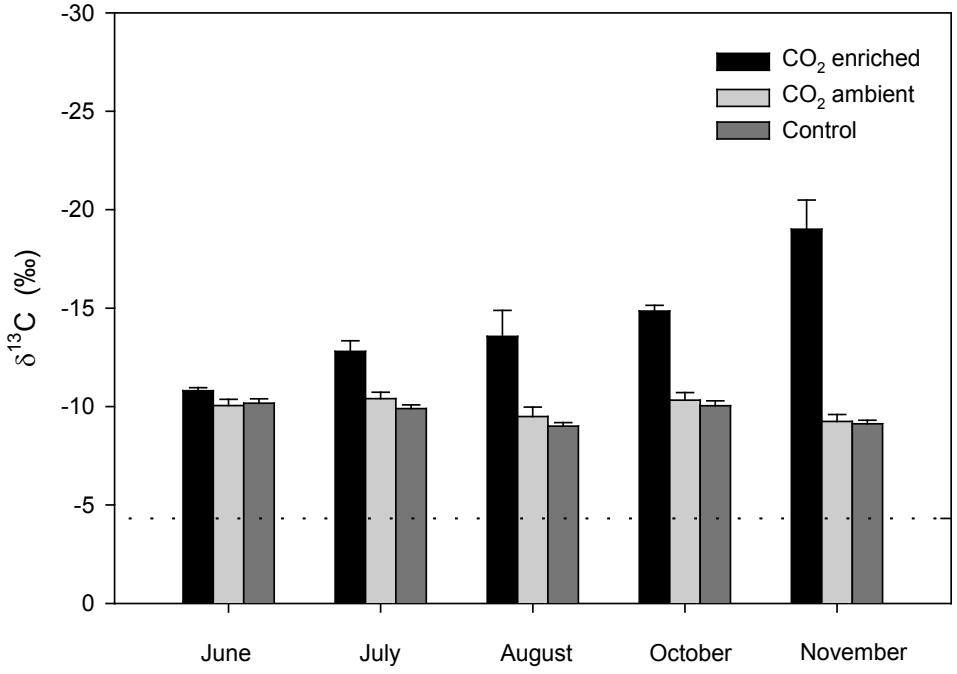

-30
-25
-20
-15
-10
-5
0
δ13C (‰)
CO2 enriched
CO2 ambient
Control
June
July
August
October
November

FIGURE 7.

CHAPTER IV

EFFECTS OF IN SITU CO_2 ENRICHMENT ON THE PRODUCTIVITY, NUTRIENT AND CARBOHYDRATE CONTENT OF THE SEAGRASS *THALASSIA TESTUDINUM*

Campbell JE, Fourqurean JW (Submitted). *In situ* CO_2 enrichment alters the nutrient and carbohydrate content of the seagrass, *Thalassia testudinum*. Marine Ecology Progress Series.

ABSTRACT

Seagrasses commonly display carbon-limited photosynthetic rates. Thus, increases in atmospheric pCO_2, and consequentially oceanic $CO_{2(aq)}$ concentrations, may prove beneficial. While *ex situ* mesocosm experimentation has provided some evidence for this, we know relatively little in regards to *in situ* responses to elevated $CO_{2(aq)}$ concentrations, stressing the importance of field experimentation. We examine the physiological effects of *in situ* $CO_{2(aq)}$ enrichment on the widely-distributed marine angiosperm, *Thalassia testudinum*. We utilize a series of clear, open-top chambers to continuously manipulate seawater carbonate parameters around a tropical seagrass bed, and replicate $CO_{2(aq)}$ forecasts for the year 2100. After 6 months, seagrass growth responses to $CO_{2(aq)}$ enrichment were relatively weak, while plant nitrogen (N) and phosphorus (P) content strongly declined, increasing leaf C:N and C:P ratios. Elevated $CO_{2(aq)}$ additionally increased non-structural carbohydrates (NSC). Our results generally validate responses from prior research, and suggest that seagrasses will likely undergo a number of physiological and ecological changes under $CO_{2(aq)}$ enrichment, potentially altering the future functionality of these systems.

INTRODUCTION

Climate change will represent a persistent and widespread stress on a variety of marine ecosystems. Anthropogenic activities, such as fossil fuel combustion and deforestation have contributed vast quantities of CO_2 to both atmospheric and oceanic reservoirs. To date, these activities have increased oceanic $CO_{2(aq)}$ concentrations, and

have reduced seawater pH by nearly 0.1 units since the industrial revolution of the 1800s (Brewer 1997). Continued declines of 0.3 -0.5 pH units, and a near 3-fold increase in $CO_{2(aq)}$ concentrations are expected over the course of the next century (Brewer 1997; Caldeira and Wickett 2005). As such, a large body of research has been directed towards studying the responses of natural systems to these rapidly changing physiochemical conditions. While research on the effects of climate change within marine ecosystems has been expanding, the majority of this work has been conducted within *ex situ* mesocosms, resulting in calls for studies which operate under increasingly realistic environmental conditions (Doney et al. 2009; Hendriks et al. 2010; Wernberg et al. 2012).

While a large number of studies document the negative impacts of ocean acidification on a variety of calcified invertebrates, research highlighting the beneficial responses of $CO_{2(aq)}$ enrichment on marine primary producers (primarily seagrasses) has become increasingly prominent (Beer and Koch 1996; Zimmerman et al. 1997; Invers et al. 2002; Palacios and Zimmerman 2007; Hall-Spencer et al. 2008; Jiang et al. 2010). Due to the relatively low concentration of $CO_{2(aq)}$ in seawater, and inefficient use of HCO_3^- as an inorganic carbon source (Durako 1993; Beer and Koch 1996; Invers et al. 2001), a large number of seagrass taxa have carbon-limited photosynthetic rates and exhibit substantial improvements in overall plant carbon balance with elevated $CO_{2(aq)}$ (Zimmerman et al. 1997; Invers et al. 2002). Alternate responses, such as increases in non structural carbohydrates (NSC) and reproductive output have been additionally documented, and can have ecological implications for the future functionality of these systems (Zimmerman et al. 1997; Palacios and Zimmerman 2007). To date, the effects of *in situ* seagrass $CO_{2(aq)}$ enrichment have received some attention; with research

documenting photosynthetic responses (Schwarz et al. 2000), changes in the epiphyte community (Martin et al. 2008), and shoot density (Hall-Spencer et al. 2008). While we have improved our understanding of how submerged plant communities might respond to additional $CO_{2(aq)}$, documentation of *in situ* responses remains scarce; with most observations coming from natural CO_2 vents rather than manipulative experiments, limiting our ability to unequivocally ascertain whether observed responses are indeed solely driven by changes in $CO_{2(aq)}$ availability.

Many coastal environments exhibit substantial variation in seawater carbonate parameters. The shallow, productive nature of these environments can produce large diurnal and seasonal swings in $CO_{2(aq)}$ concentrations, partially attributable to the presence of pelagic and benthic biological communities (Yates et al. 2007). As some coastal regions already have diurnal pH variation as high ± 0.2 units, continued ocean acidification may serve to further drive down mean pH values, and dramatically increase $CO_{2(aq)}$ concentrations within these areas (Wootton et al. 2008). As oceanic $CO_{2(aq)}$ concentrations are forecast to nearly triple by the end of this century (Brewer 1997), it is important to replicate not only expected increases in $CO_{2(aq)}$, but additionally mimic both diurnal and seasonal shifts in the seawater carbonate parameters over long time scales (Hofmann et al. 2011). Thus, *in situ* experimentation can ideally serve to replicate natural ocean acidification scenarios, and further advance our understanding of how submerged plant communities respond to additional $CO_{2(aq)}$.

Terrestrial research has detailed a number of varying physiological and ecological effects of CO_2 enrichment on overall plant functioning and performance; suggesting that similar responses might exist for submerged plants. While short-term photosynthetic

responses are well established; long-term terrestrial ecological responses are more nuanced and can be governed by the availability of alternate resources or a number of environmental factors. For example, elevated CO_2 can increase NSC in a wide variety of plants, however these responses have shown interspecific and condition dependent variation (Stitt 1991; Korner et al. 1995; Poorter et al. 1997; Tissue et al. 1997; Stitt and Krapp 1999). Nitrogen limitation can promote NSC accumulation in CO_2 enriched plants (Wong 1980; Wong 1990; Bowler and Press 1996; Baxter et al. 1997) and potentially constrain a number of large-scale ecosystem responses (Oren et al. 2001; Reich et al. 2006). Furthermore, terrestrial studies have documented declines in overall plant nitrogen content with elevated CO_2, potentially attributable to either carbohydrate dilution, imbalances between nutrient supply and demand, or photosynthetic downregulation of nitrogen rich compounds (Stitt and Krapp 1999). These trends can have important ramifications for a number of ecosystem properties, and potentially influence higher trophic levels (Bazzaz 1990; Bezemer and Jones 1998). While receiving limited attention under laboratory settings, the effects of CO_2 enrichment on seagrass nutrient status have not been studied under natural field conditions.

Seagrass meadows play an important role in organic carbon production and nutrient cycling in many coastal regions around the world (Orth *et al.* 2006). The importance of seagrass meadows as sites of carbon sequestration is now being realized, as coastal meadows account for nearly 15% of all oceanic production, and roughly 50% of carbon burial (Duarte and Chiscano 1999). Seagrass ecosystem carbon (C) storage rivals the storage of C in terrestrial forests and mangrove ecosystems on an areal basis, as most of the C storage is in the sediment organic matter (Fourqurean et al. 2012). Furthermore,

the export of fixed carbon from seagrass ecosystems substantially contributes to the carbon budget of adjacent ecosystems (Heck et al. 2008), thus understanding the ecological implications of CO_2 related changes in the functionality (particularly as it relates to biomass production) of these coastal systems remains an important topic.

This study employed a new technique of *in situ* experimentation (Campbell and Fourqurean 2011) to evaluate how additional $CO_{2(aq)}$ influences the physiological and ecological properties of the tropical seagrass *Thalassia testudinum*. Furthermore, we expanded the scope of prior work by documenting the responses of a widespread tropical species, an often underrepresented taxa in seagrass CO_2 research. We manipulated $CO_{2(aq)}$ concentrations in a shallow seagrass meadow for a period of 6 months, and evaluated responses in productivity, nutrient status, and carbohydrate storage. We hypothesized that additional $CO_{2(aq)}$ would significantly increase both seagrass productivity and carbohydrate content over the course of the experiment. While the effects of $CO_{2(aq)}$ enrichment on nutrient dynamics have received limited attention, we further expected plant nutrient content to decline with additional $CO_{2(aq)}$, as indicated in prior mesocosm research (Jiang et al. 2010). The conclusions of this study provide an important link between *ex situ* experimentation and the *in situ* responses of naturally occurring seagrass beds, advancing our understanding of how short-term physiological responses scale into long-term ecological impacts.

METHODS

Site description

Benthic *in situ* CO_2 enrichment was conducted within a shallow (1m depth), sub-

tidal seagrass meadow in the Florida Keys, Florida, USA (24.55^{o} N, 81.75^{o} W). The benthic community was dominated by the seagrass *Thalassia testudinum*, with lower abundances of the seagrasses *Syringodium filiforme* and *Halodule wrightii*. The sediments were comprised of mud-sized biogenic calcium carbonates consisting of approximately 10% organic matter. During the experiment, noon light levels at the top of the seagrass canopy averaged 800 *umol* photons m^{-2} s^{-1}, while salinity averaged 37. Seawater temperatures were recorded every 6 hrs with a HOBO temperature logger. Temperatures were highest during July, August, September, and October; averaging 30.9°C and ranging from 22.5°C - 34.5°C. During the last two months of November and December, temperatures averaged 24.9°C and 23.4°C respectively, and ranged from 17.2°C - 29.7°C.

In situ $CO_{2(aq)}$ enrichment

The $CO_{2(aq)}$ enrichment was conducted continuously for 6 months (July 1, 2009 - January 1, 2010). Carbon dioxide enriched seawater was pumped into a series of optically-clear, open-top acrylic chambers which were anchored within the seagrass meadow (see Campbell & Fourqurean 2011 for detailed description of the experimental set-up). Three replicated treatments (n = 5) were arranged in a randomized grid design; 1) elevated $CO_{2(aq)}$ within chambers, 2) ambient $CO_{2(aq)}$ within chambers, and 3) unmanipulated, open control plots. Each of the chambered treatments was connected to a submerged PVC network and supplied with either ambient seawater or $CO_{2(aq)}$ rich seawater from a series of water pumps. $CO_{2(aq)}$ enrichment was carefully controlled, and set to mimic $CO_{2(aq)}$ forecasts for the year 2100, roughly a 0.4 unit reduction in pH

(Brewer 1997; Caldeira and Wickett 2005). Total alkalinity and pH within each chamber was periodically monitored for the duration of the experiment. Fouling on the surface of the chambers was removed on a weekly basis. Seawater carbonate parameters (Table1) were calculated with the CO_2Sys Excel macro (Lewis and Wallace 1998), using the dissociation constants of (Mehrbach et al. 1973), refit by (Dickson and Millero 1987).

Seagrass growth, shoot characteristics and standing crop

Leaf growth rates were measured on a monthly basis (with the exception of October) following a modified hole-punch methodology (Zieman 1974). Within each chamber and open plot, all the leaves of 3 - 5 short shoots of *T. testudinum* were pierced with a hypodermic needle near the base. Marked shoots were then harvested after 7 - 10 days of *in situ* growth. In the lab, all epiphytes were gently removed with a razor blade, and the lengths and widths of all leaves were recorded. Each shoot was then divided into newly produced leaf material and older leaf material and dried to a constant weight in a 70°C oven. Relative leaf growth rates for each shoot were calculated as the mass of new leaf growth per existing shoot mass (mg g^{-1} d^{-1}). Absolute leaf growth per shoot (mg $shoot^{-1}$ d^{-1}) and total shoot mass (dry g $shoot^{-1}$) were additionally calculated. Seagrass density (no. shoots m^{-2}) was recorded on a monthly basis by counting all shoots within 2 haphazardly placed10 x 15 cm quadrats within each chamber and open plot. Seagrass standing crop for each month was calculated by multiplying the average shoot mass by the recorded shoot density for each chamber and open plot.

Seagrass nutrient content

Nutrient content of seagrass leaf material was measured in each chamber and

control plot on a monthly basis (with the exception of December). Newly produced leaf material (leaf rank 1) from 3-5 shoots was analyzed for carbon (C), nitrogen (N), and phosphorus (P) content (% g dry wt). Entire shoots (new + old leaf material) were additionally analyzed at the beginning of the experiment to facilitate comparisons of nutrient content with other seagrass studies conducted in South Florida. Dried leaf material was ground into a fine powder with a mortar and pestle, and analyzed for C and N content using a CHN analyzer. P content of dried leaf material was determined via dry oxidation, acid hydrolysis extraction followed by colorimetric analysis (Fourqurean et al. 1992a). Elemental ratios are reported on a mole : mole basis.

Seagrass non-structural carbohydrate content

Non-structural carbohydrate content (NSC) of belowground seagrass material (roots and rhizomes) was determined utilizing the MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) analysis method (Johnson et al. 1981; Pakulski and Benner 1992). At the conclusion of the experiment in January, 3-5 shoots of *T. testudinum* were carefully harvested from each chamber and control plot. In the lab, non-photosynthetic portions of the vertical short shoot and roots were separated from aboveground leaf material, dried to a constant weight at 60^{o}C, and ground to a fine powder. Chemical analysis of the dried belowground material (vertical rhizomes and roots separately) involved a borohydride reduction of hydrolyzed monosaccharides to sugar alcohols, followed by periodate oxidation to formaldehyde, and colorimetric determination of formaldehyde by MBTH (Lee and Dunton 1997). NSC is reported as mg soluble carbohydrate g^{-1}dry mass.

Statistical analyses

Seagrass leaf growth, shoot characteristics, and nutrient content were analyzed using a repeated-measures analysis of variance (ANOVA, $\alpha = 0.05$), with month as the within-subject factor, and CO_2 treatment (unmanipulated open plots, ambient $CO_{2(aq)}$ chambers, and $CO_{2(aq)}$ enriched chambers) as the between subject factor. When significance was detected, post-hoc analysis was conducted with a Holm-Sidak test at an overall significance level of 0.05. To avoid pseudoreplication, statistical analyses were conducted utilizing the means of replicate subsamples from within each chamber or open plot. All data were tested for normality and variance homogeneity. When such tests were violated, data were either log transformed, or ranked and analyzed non-parametrically. Rhizome and root NSC were analyzed with a one-way analysis of variance. All statistical analyses were performed with SigmaStat 11.0 (Systat software).

RESULTS

$CO_{2(aq)}$ enrichment carbonate parameters

In situ $CO_{2(aq)}$ enrichment was effectively maintained within the shallow seagrass meadow for the duration of the experiment. Seawater carbonate parameters were significantly altered within the enriched chambers as compared to the control chambers and plots (Table 1). There were no significant differences in carbonate parameters between the ambient chambers and the open plots. Mean pH values within the enriched chambers, ambient chambers, and open plots were 7.78, 8.23, and 8.24, respectively. On average, $CO_{2(aq)}$ concentrations were increased by 3-fold within the enriched chambers as

compared to the controls (see Campbell and Fourqurean (2011) for a full description of all carbonate parameters).

Seagrass growth, shoot characteristics and standing crop

Elevated $CO_{2(aq)}$ had no effect on seagrass shoot mass, shoot density, shoot leaf area, leaf growth rates or standing crop (Figs 1,2; Table 2). Shoot mass, shoot leaf area, and standing crop all significantly declined during the enrichment period in all treatments, following the normal seasonality observed in South Florida seagrass communities (Fourqurean et al. 2001) Across carbon treatments, mean shoot mass (± 1SE) ranged from 0.42 ± 0.02 g shoot^{-1} in July to 0.23 ± 0.01 g shoot^{-1} in December. Mean leaf area (± 1SE) similarly displayed highest values in July (90.3 ± 3.43 cm^2 shoot^{-1}) and lowest values in December (53.2 ± 2.51 cm^2 shoot^{-1}), as did standing crop (ranging from 290.8 ± 24.8 to 134.8 ± 11.4 g m^{-2}). Mean shoot density (± 1SE) ranged from a minimum of 520.4 ± 39.3 shoots m^{-2} in August to a maximum of 748.4 ± 42 shoots m^{-2} in November. Mean relative leaf growth rates (± 1SE) were highest in November (22.0 ± 0.7 mg g^{-1} d^{-1}) and lowest in December (16.6 ± 0.7 mg g^{-1} d^{-1}). Absolute leaf growth rates (mg shoot^{-1} d^{-1}) followed similar trends (data not shown). Significant interactions between $CO_{2(aq)}$ and time were not detected.

Seagrass nutrient content

Seagrass nutrient content displayed significant variation over time and with $CO_{2(aq)}$ enrichment (Fig. 3;Table 2). Averaged across treatment, leaf N content ranged from a minimum of 2.04 ± .05% (mean ± 1SE) in July to a maximum of 2.53 ± .06% in September. C:N ratios were lowest during the months of August and September, and

highest at the first sampling in July. Comparisons among carbon treatments revealed that leaf N content was lowest, and leaf C:N ratios were highest within the $CO_{2(aq)}$ enriched chambers, as compared to the ambient chambers and control plots.

While leaf P content did not display significant variation over time, leaf C:P ratios did significantly vary. Across all carbon treatments, mean leaf C:P ratios (± 1SE) ranged from a minimum of 580.4 ± 30.9 in July to a maximum of 703.6 ± 32.9 in October. Comparing $CO_{2(aq)}$ treatments, leaf %P was lowest, and leaf C:P ratios were highest in the $CO_{2(aq)}$ enriched chambers. Leaf N:P ratios followed similar trends and were significantly impacted by time and $CO_{2(aq)}$ enrichment. N:P ratios displayed a treatment average of 31.3 ±1.0 in July to a maximum of 40.5 ± 1.7 in September. Treatment comparisons show that leaf N:P ratios were elevated within the $CO_{2(aq)}$ enriched chambers as compared to the ambient chambers and control plots. Significant interactions between $CO_{2(aq)}$ treatment and time were not detected for any nutrient response variable.

Seagrass non-structural carbohydrates (NSC)

Seagrass non-structural carbohydrate content responded to $CO_{2(aq)}$ enrichment, with higher concentrations (means ± 1SE) within the enriched chambers (222.7 ± 11.1 mg C g^{-1}) as compared to the ambient chambers (172.7 ± 4.5 mg C g^{-1}) and open plots (171.0 ± 10.1 mg C g^{-1}) (Fig. 4, Table 2). Root NSC content was significantly lower than rhizome NSC content, and did not display and significant differences among treatments.

DISCUSSION

This study documents the responses of a natural seagrass bed to *in situ* $CO_{2(aq)}$ enrichment and supports various findings from prior mesocosm research. While responses in leaf growth rates were not detected, increases in short shoot NSC were observed under $CO_{2(aq)}$ enrichment, suggesting that increased allocation towards belowground structures might represent a widespread response of seagrass communities to ocean acidification. While $CO_{2(aq)}$ mediated responses in seagrass nutrient content have received limited attention, we observe declines in leaf N and P content (% dry mass), consistent with prior work (Jiang et al. 2010). This study demonstrates that: 1) Because of NSC buildup, $CO_{2(aq)}$ mediated increases in photosynthetic rates might not translate into elevated growth over short time scales, and 2) observed shifts in leaf nutrient content indicate a potential link between the availability of mineral resources and seagrass responses to carbon enrichment.

Relative leaf growth rates were comparable to values previously reported for *T. testudinum* in South Florida (Fourqurean et al. 2001). However, this study detected maximum leaf growth rates in November, distinct from typical summer peaks in July and August (Fourqurean et al. 2001). Temperature records indicate that seawater temperatures chronically exceeded thermal optima (ca. 30°C) for this species during the summer months of the present study (peaking at 34.5°C). The elevated growth rates in November likely reflect cooler seawater temperatures (ranging from 22 - 29°C), and thus a reduction in thermal stress in *T. testudinum*. Temporal declines in seagrass shoot characteristics (shoot mass, leaf area, and standing crop) across all carbon treatments during the course

of this study likely represent the influence of seasonal variation in the light environment on seagrass growth, as suggested for several species (Duarte 1989; Marba et al. 1996; Fourqurean et al. 2001). Relative leaf growth rates did not respond to $CO_{2(aq)}$ enrichment, similar to findings from prior mesocosm research (Palacios and Zimmerman 2007). Any indication that rates were trending towards higher values within the enriched chambers at the beginning of the experiment were transient. The overall insensitivity of seagrass leaf growth rates to elevated $CO_{2(aq)}$ may be the result of several factors; however our results indicate that over the timeframe of this experiment, increased allocation of resources towards belowground structures may have occurred at the expense of increased shoot growth. Contrary to Palacios and Zimmerman (2007), shoot proliferation was not increased by $CO_{2(aq)}$ enrichment, which could be related to experimental duration. The present study manipulated $CO_{2(aq)}$ levels for 6 months, whereas Palacios and Zimmerman (2007) only detected significant differences in shoot proliferation after a year of enrichment, indicating variation in time-dependent responses of seagrass communities to $CO_{2(aq)}$ enrichment. Studies near volcanic vents document increases in seagrass shoot density under $CO_{2(aq)}$ enrichment (Hall-Spencer et al. 2008), suggesting that short-term responses to carbon enrichment are likely distinct from long-term responses. Furthermore, enrichment had no effect on seagrass leaf area or aboveground shoot mass, thus the impacts of $CO_{2(aq)}$ on seagrasses might be poorly represented at the level of individual shoots, as suggested by Palacios and Zimmerman (2007).

The allocation of carbohydrates towards belowground rhizomes may account for the weak growth responses of *T. testudinum*. Non-structural carbohydrates can often be directed towards a number plant compartments, and many perennial species must

navigate tradeoffs between investing resources in current growth (leaf or root expansion), or storing these resources for mobilization at a later date. While root growth was not estimated, our documented $CO_{2(aq)}$ mediated increases in rhizome NSC content follows prior research (Zimmerman et al. 1997; Jiang et al. 2010), and suggests that a certain proportion of increased NSC production may be directed towards rhizome storage as opposed to leaf growth. Many seagrasses are perennial and have the ability to accumulate storage compounds when carbohydrate supply exceeds demand; and as similar to terrestrial systems, these source/sink imbalances strongly regulate the extent of carbohydrate storage (Korner 2003). Storage capacity confers an advantage upon vegetation which experiences fluctuating environmental conditions (i.e., prolonged shading events or biomass removal from herbivores), thus NSCs can serve as important substrates to support future plant respiration or replace lost tissue (Chapin et al. 1990). We suggest that experiments directed towards evaluating the ability of $CO_{2(aq)}$ enriched seagrasses to endure disturbance events is warranted by the conclusions of both *ex* and *in situ* studies.

The nutrient content of *T. testudinum* at the beginning of this study was comparable to values documented in prior regional assessments of seagrass nutrient content across South Florida. Both leaf %N and C:N ratios were similar to averages reported in a large-scale, multiyear survey of seagrass nutrient content in South Florida (Fourqurean and Zieman 2002). Furthermore, leaf % P was slightly lower, and both C:P and N:P ratios were slightly higher than reported averages. Under *in situ* $CO_{2(aq)}$ enrichment, the nutrient content of *T. testudinum* strongly decreased, following trends documented by prior seagrass (Jiang et al. 2010) and terrestrial plant research (Cotrufo et

al. 1998). Similar to these studies, declines in leaf N and P content may have resulted from a rapid dilution of the current nutrient pool by NSC buildup (Stitt and Krapp 1999). The observation that both leaf N and P content were concurrently reduced is further indicative of mineral dilution by NSCs, and not likely attributable to a physiological downregulation of photosynthetic machinery (Gifford et al. 2000). Furthermore, research with the temperate seagrass *Zostera marina* documented no evidence of photosynthetic downregulation, nor any decline in photosynthetic performance over the course of a 45 day $CO_{2(aq)}$ enrichment period (Zimmerman et al. 1997).

Alternative to NSC dilution, these observations suggest that nutrient supply may have been unable to match temporary increases in leaf growth, resulting in leaf material with higher C content relative to N and P. Thus, the availability of mineral resources might play a role in the ability of plants to respond to additional $CO_{2(aq)}$ via increased growth, as demonstrated for some terrestrial systems (Bazzaz 1990; Reich et al. 2006). Under nutrient-limited conditions, long-lived seagrasses are constrained in their ability to produce additional leaf biomass, and might alternatively increase NSC allocation towards belowground rhizomes, as suggested by prior correlative field surveys (Campbell et al. 2012). These trends suggest that inadequate nutrient supply may have promoted NSC storage in lieu of leaf growth for our enriched seagrasses. While our experimental design precludes us from making strong inferences in regards to the role that nutrient availability plays in regulating seagrass responses to $CO_{2(aq)}$, we suggest this as a possible mechanism to consider for future work.

In situ experimentation is critical to fully evaluate conclusions derived from prior laboratory and mesocosm research. Our manipulative field study provides a powerful

experimental design through which we can evaluate the responses of a benthic community to natural ocean acidification scenarios. The responses of *T. testudinum* to 6 months of $CO_{2(aq)}$ enrichment follow some of the trends detected in prior laboratory and mesocosm research (Zimmerman et al. 1997; Palacios and Zimmerman 2007; Jiang et al. 2010), primarily demonstrating impacts on the chemical composition of both aboveground and belowground components of seagrass systems. Documented shifts in leaf nutrient content suggest that the availability of mineral resources might play a role in the response of seagrasses to additional $CO_{2(aq)}$. Furthermore, changes in nutrient content and overall chemical composition may have implications for direct grazing of leaf tissue, as documented in some terrestrial systems (Bazzaz 1990; Bezemer and Jones 1998). Lastly, increases in rhizome NSC may have implications for seagrass resilience, potentially altering the ability of these plants to endure disturbance events (i.e. prolonged shading or extensive herbivory). The long term fate of this additional NSC remains unknown, and might ultimately depend upon alternate environmental factors (Mooney et al. 1995). Thus, tracking $CO_{2(aq)}$ mediated seagrass responses across a number of environmental gradients (such as light and nutrient availability) over long time scales will improve our understanding of how ocean acidification impacts these widely-distributed systems.

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Table 1. Mean seawater carbonate parameters (plus 95% confidence intervals) calculated at noon within the seagrass canopy throughout the 6 month enrichment period. Total alkalinity and pH were measured *in situ*, all other carbonate parameters were calculated with the CO_2Sys Excel Macro, using the carbonic acid dissociation constants of Mehrbach et al. (1973), refit by Dickson and Millero (1987). See Campbell and Fourqurean (2011) for a detailed description of all carbonate parameters.

Treatment	pH	DIC (µmol/kg SW)	CO_2 (µmol/kg SW)	HCO_3^- (µmol/kg SW)	CO_3^{2-} (µmol/kg SW)
CO_2 enriched	7.78 (7.72-7.84)	2168 (2042-2294)	32.2 (25.6-38.9)	2013 (1892-2133)	123.4 (110.8-136.0)
CO_2 ambient	8.23 (8.17-8.29)	1919 (1786-2051)	8.8 (6.9-10.7)	1636 (1498-1774)	274.4 (246.5-302.3)
Control	8.24 (8.18-8.30)	1917 (1783-2050)	8.7 (6.8-10.7)	1632 (1493-1771)	276.0 (248.2-303.7)

Table 2. *Thalassia testudinum*. Results of repeated measures ANOVA on metrics of seagrass production and chemical composition. Significant results are indicated in bold. Post hoc results (Holm-Sidak, $\alpha = 0.05$) are only indicated for the carbon treatment.

	Source of variation			Posthoc results
	Carbon	Time	Carbon x Time	Carbon
Shoot mass (g shoot^{-1})	F = 0.17 , P = 0.847	**F = 24.21 , P < 0.001**	F = 0.53 , P = 0.823	
Shoot leaf area (cm^2 shoot^{-1})	F = 1.14 , P = 0.351	**F = 11.15 , P < 0.001**	F = 0.39 , P = 0.922	
Shoot density (# m^{-2})	**F = 5.06 , P < 0.026**	**F = 7.38 , P < 0.001**	F = 0.90 , P = 0.527	No CO_2 > Open
Standing crop (g m^{-2})	F = 1.99 , P = 0.180	**F = 14.97 , P < 0.001**	F = 1.23 , P = 0.305	
Relative leaf productivity (mg g^{-1} d^{-1})	F = 1.38 , P = 0.290	**F = 6.78 , P < 0.001**	F = 0.52 , P = 0.835	
Absolute leaf productivity (mg shoot^{-1} d^{-1})	F = 0.25 , P = 0.782	**F = 13.74 , P < 0.001**	F = 0.66 , P = 0.72	
Leaf carbon content (% DW)	F = 0.08 , P = 0.924	**F = 65.13 , P < 0.001**	F = 1.68 , P = 0.154	
Leaf nitrogen content (% DW)	**F = 12.15 , P = 0.001**	**F = 10.83 , P < 0.001**	F = 0.21 , P = 0.972	No CO_2 > CO_2 ,Open
Leaf phosphorus content (% DW)	**F = 12.75 , P = 0.001**	F = 1.67 , P = 0.191	F = 0.18 , P = 0.980	No CO_2 > CO_2 ,Open
Leaf C:N	**F = 10.59 , P = 0.002**	**F = 11.99 , P < 0.001**	F = 0.176 , P = .982	CO_2 > No CO_2 , Open
Leaf C:P	**F = 8.87 , P = 0.004**	**F = 3.60 , P = 0.004**	F = 0.33 , P= 0.915	CO_2 > No CO_2
Leaf N:P	**F = 4.47 , P = 0.035**	**F = 8.725 , P < 0.001**	F = 0.59 , P = 0.738	CO_2 > No CO_2
Rhizome soluble carbohydrates (mg C g^{-1} DW)	**F = 8.14 , P < 0.01**	—	—	CO_2 > No CO_2, Open
Root soluble carbohydrates (mg C g^{-1} DW)	F = 0.479 , P = 0.631	—	—	

FIGURE CAPTIONS

Figure 1. *Thalassia testudinum*. Shoot mass, shoot density, shoot leaf area, and standing crop (means ± 1 SE) in the enriched chambers, ambient chambers and open plots.

Figure 2.*Thalassia testudinum.* Relative leaf growth (means ± 1 SE) in the enriched chambers, ambient chambers, and open plots.

Figure 3. *Thalassia testudinum*. Leaf C:N, C:P, and N:P ratios (means ± 1 SE) in the enriched chambers, ambient chambers, and open plots. Asterisks indicate significant results of post hoc analyses within each sampling event (Holm-Sidak, $\alpha = 0.05$).

Figure 4. *Thalassia testudinum*. Non-structural carbohydrate content (means ± 1 SE) of belowground rhizomes and roots in the enriched chambers, ambient chambers, and open plots. Distinct letters indicate significant differences from post hoc analysis (Holm-Sidak, $\alpha = 0.05$)

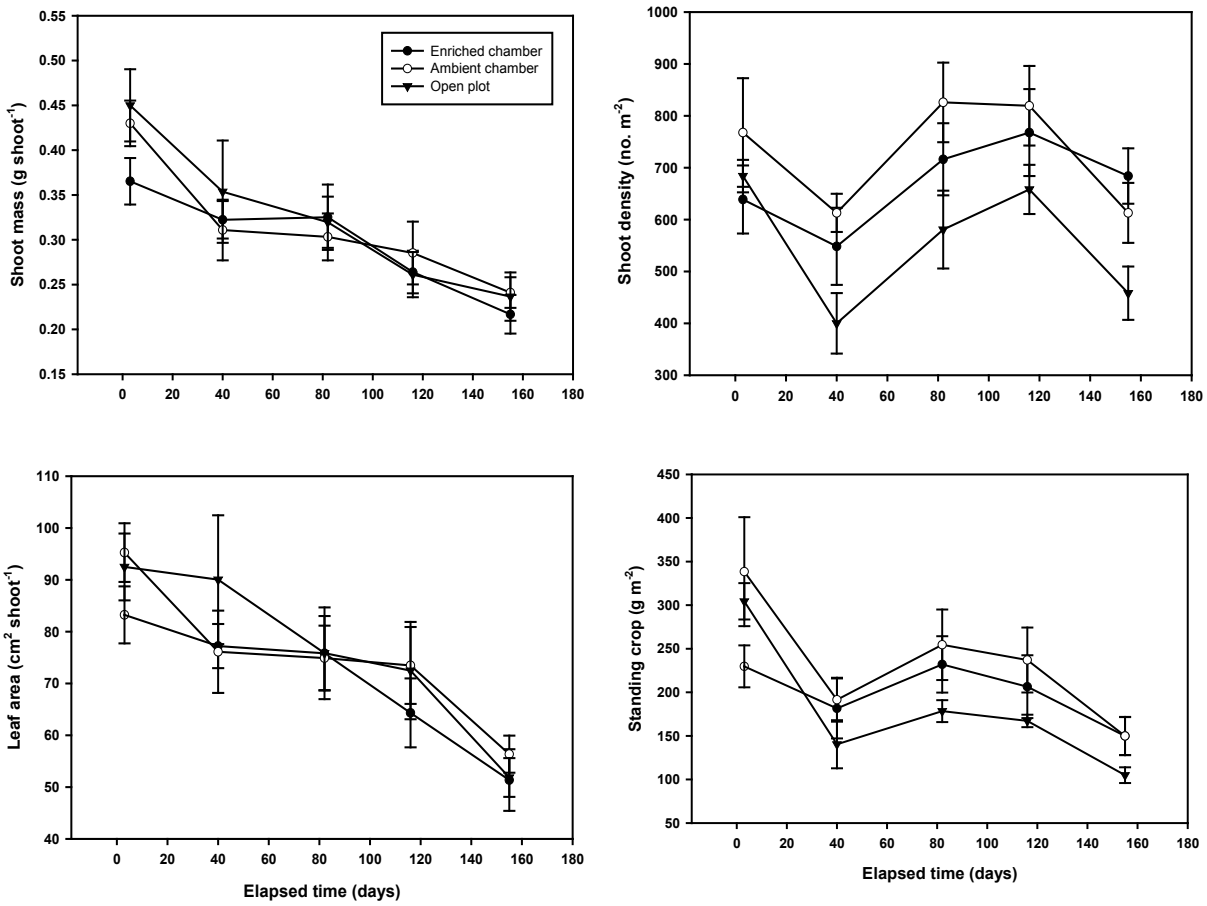

Enriched chamber
Ambient chamber
Open plot
Shoot mass (g shoot-1)
Shoot density (no. m-2)
Leaf area (cm2 shoot-1)
Standing crop (g m-2)
Elapsed time (days)
Elapsed time (days)

FIGURE 1.

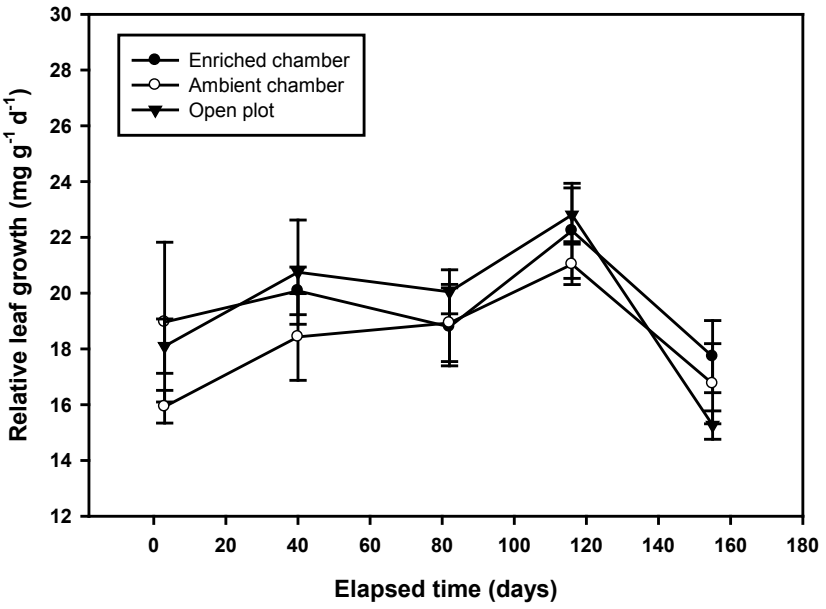
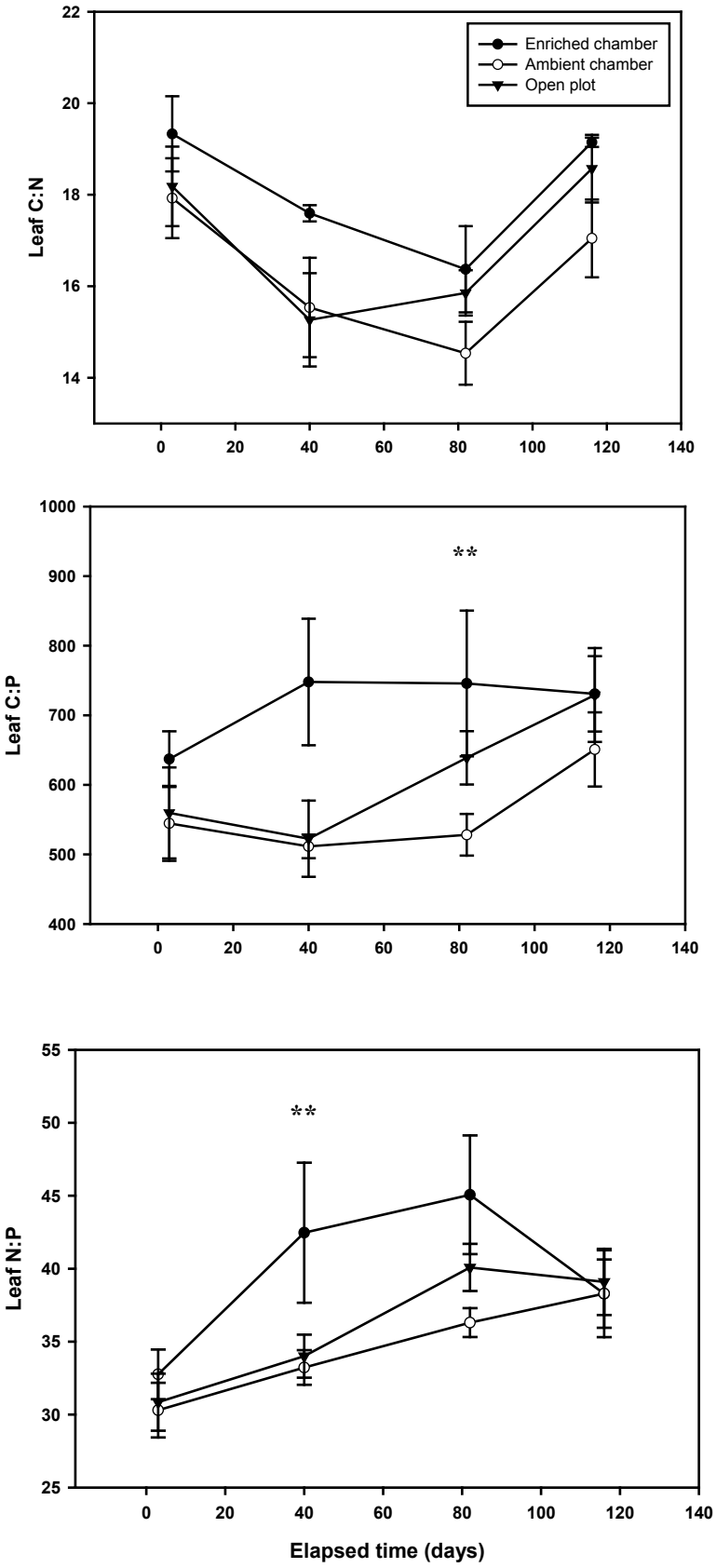

Enriched chamber
Ambient chamber
Open plot
Relative leaf growth (mg g-1 d-1)
Elapsed time (days)
30
28
26
24
22
20
18
16
14
12
0
20
40
60
80
100
120
140
160
180

FIGURE 2.


Enriched chamber
Ambient chamber
Open plot
Leaf C:N
Leaf C:P
Leaf N:P
**
**
Elapsed time (days)

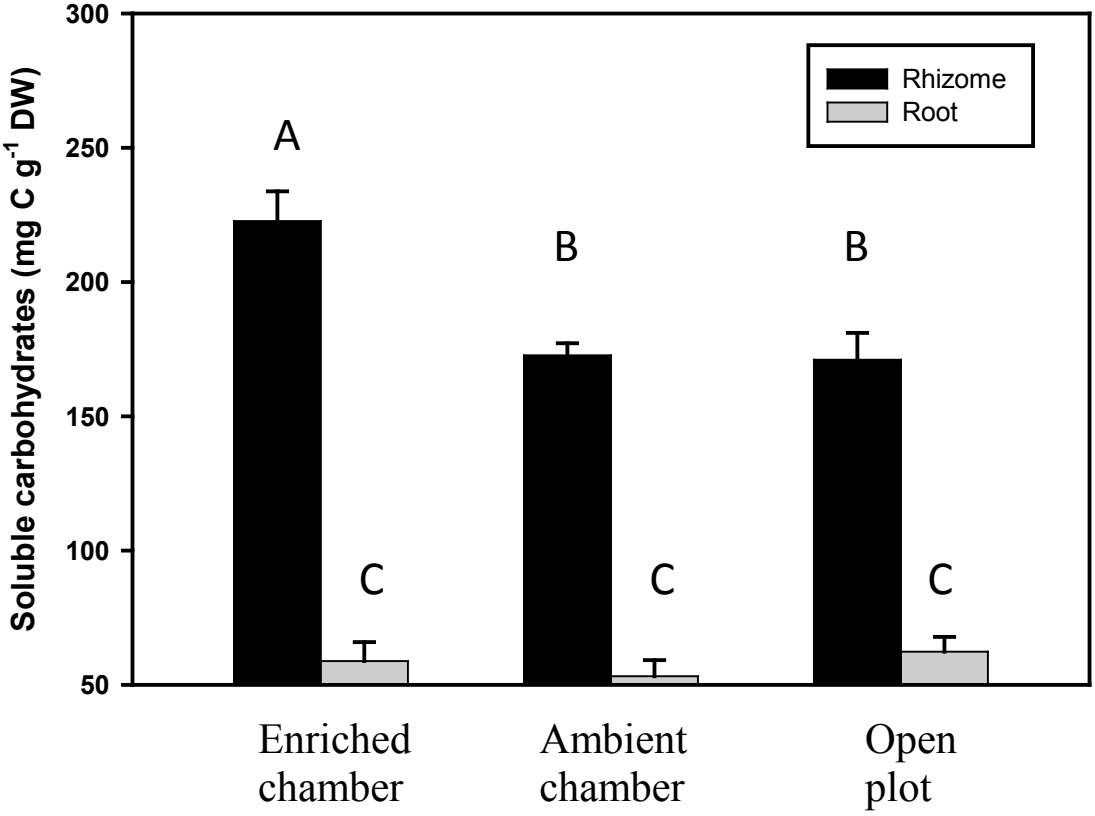

Soluble carbohydrates (mg C g-1 DW)
300
250
200
150
100
50
Rhizome
Root
A
B
B
C
C
C
Enriched chamber
Ambient chamber
Open plot

FIGURE 4.

CHAPTER V

NEGATIVE RELATIONSHIPS BETWEEN THE NUTRIENT AND CARBOHYDRATE CONTENT OF THE SEAGRASS *THALASSIA TESTUDINUM*

Campbell JE, Fourqurean JW (2012) Negative relationships between the nutrient and carbohydrate content of the seagrass *Thalassia testudinum*. Aquatic Botany 99:56-60

ABSTRACT

This study documents relationships between plant nutrient content and rhizome carbohydrate content of a widely distributed seagrass species, *Thalassia testudinum*, in Florida**.** Five distinct seagrass beds were sampled for leaf nitrogen, leaf phosphorus, and rhizome carbohydrate content from 1997-1999. All variables displayed marked intra- and inter- regional variation. Elemental ratios (mean N:P ± S.E.) were lowest for Charlotte Harbor (9.9 ± 0.2) and highest for Florida Bay (53.5 ± 0.9), indicating regional shifts in the nutrient content of plant material. Rhizome carbohydrate content (mean ± S.E.) was lowest for Anclote Keys (21.8 ± 1.6 mg g^{-1}FM), and highest for Homosassa Bay (40.7 ± 1.7 mg g^{-1}FM). Within each region, significant negative correlations between plant nutrient and rhizome carbohydrate content were detected; thus, nutrient-replete plants displayed low carbohydrate content, while nutrient-deplete plants displayed high carbohydrate content. Spearman's rank correlations between nutrient and carbohydrate content varied from a minimum in Tampa Bay (ρ =-0.2) to a maximum in Charlotte Harbor (ρ =-0.73). Linear regressions on log-transformed data revealed similar trends. This consistent trend across five distinct regions suggests that nutrient supply may play an important role in the regulation of carbon storage within seagrasses. Here we present a new hypothesis for studies which aim to explain the carbohydrate dynamics of benthic plants.

INTRODUCTION

The ability of plants to endure disturbance events strongly depends upon their capacity to support essential growth and maintenance functions during unfavorable environmental conditions. Non-structural carbohydrate (NSC) reserves play an important role in the resilience of perennial plants by serving as a "rescue mechanism", allowing plants to sustain respiration or rebuild damaged tissue in response to disturbance (Mcpherson and Williams 1998; Landhausser and Lieffers 2002; Poorter et al. 2010). Thus, understanding the dynamics of carbon storage, and the factors which influence carbohydrate reserves in plants may help elucidate their potential for resilience in disturbance- prone environments.

Non-structural carbohydrates build within plant storage organs by two distinct processes: true reserve formation and reserve accumulation (Chapin et al. 1990). The former process involves a metabolically regulated formation of storage carbohydrates at the expense of current plant growth, while the latter process results in a passive buildup of carbohydrates due to environmental factors (i.e., water and/or nutrient limitation) which constrain growth and reduce carbon demand (Chapin et al. 1990). Thus, the availability of external resources can strongly regulate storage dynamics, particularly in the latter case of reserve accumulation. In terrestrial plants, declines in nutrient availability can inhibit the production of new biomass, and increase stores of non-structural carbohydrates (Mooney et al. 1995; Wyka 2000; Knox and Clarke 2005). While the dynamics of carbohydrate storage have been studied for some marine plants,

few studies have addressed the role that nutrients might play in regulating storage reserves.

Seagrasses allocate a substantial portion of their biomass to belowground storage organs (rhizomes), and like terrestrial plants, these structures serve as a carbohydrate reserve to support plant growth and maintenance during periods of low photosynthetic capacity (either because of shading events or losses to herbivory). The extensive allocation of biomass to belowground structures suggests that these organs play an important role in the carbon dynamics of these plants, and may similarly be subjected to the processes of reserve accumulation.

The present study examines the relationship between plant nutrient content and rhizome carbohydrate content in the seagrass, *Thalassia testudinum*, across multiple spatial scales in Florida. Herein we present preliminary observational data to suggest that, in addition to other abiotic factors, nutrients may play an important role in regulating the size of carbohydrate reserves. Due to reserve accumulation, we hypothesized that nutrient-poor seagrasses would display increased rhizome carbohydrate content as compared to nutrient replete seagrasses. Nutrient-carbohydrate relationships were examined by sampling *T. testudinum* within five spatially distinct regions in Florida, and quantifying both the seagrass nutrient content and rhizome carbohydrate content across multiple years. Our observations suggest that future studies may need to consider the process of reserve accumulation, and the role of nutrient availability in the regulation of carbohydrate reserves.

METHODS

Study site and sampling

Five distinct regions in Florida were selected to examine the relationship between nutrient and carbohydrate content of *T. testudinum*: Homosassa Bay (N28° 45', W82° 44'), Anclote Keys (N28°12', W82°47'), Tampa Bay (N27°40', W82°42'), Charlotte Harbor (N26°48', W82°08') and Florida Bay (N24°58', W80°50'). Within each region, 30 spatially-distributed, randomly-selected points (distributed over a 0.25 km^2 grid) were sampled during the summer seasons of 1997, 1998, and 1999 (see Carlson et al., 2003 for general description). At each sampling point, intact seagrass shoots were collected using a single, 6-inch diameter sediment core, transported back to the lab on ice, and frozen until further chemical analyses. Secchi depth, temperature, and salinity were additionally recorded at each site. To quantify water clarity, the ratio between Secchi depth and site depth (Secchi ratio) was calculated; thus values near unity indicate conditions whereby the Secchi disc was visibly resting on the bottom. A portion of these data were previously used to assess regional indicators of seagrass health (Carlson et al. 2003), and examine large-scale patterns in relative nutrient availability across the eastern Gulf of Mexico (Fourqurean and Cai 2001). The analyses presented herein are novel applications of these data.

Plant chemical analysis

Seagrass shoots were washed free of sediment, and separated into aboveground and belowground material. Leaf material was gently cleaned of epiphytes using a razor blade, dried to a constant weight at 80°C, and ground to a fine powder. Carbon (C) and

nitrogen (N) content of leaf material was analyzed in duplicate using a CHN analyzer (Fisons NA1500). Leaf phosphorus (P) content was determined through a dry oxidation, acid hydrolysis extraction followed by a colorimetric analysis (Fourqurean et al. 1992a). All elemental ratios were calculated on a mole : mole basis. Belowground, rhizome non-structural carbohydrate content (sucrose and hexose) was determined using sequential extraction methods (Zimmerman et al. 1995).

Statistical methods

Spearman's rank correlation and standard linear regression were used to test the strength of the relationship between seagrass leaf nutrient content and rhizome carbohydrate content across all sampling years. Linear regressions on log transformed data were produced for the nutrient (either N or P) which provided the highest correlation with rhizome carbohydrate content for each respective region. Residuals from all linear regressions were tested for normality with a non-parametric Kolmogorov-Smirnov test (a=0.05).

RESULTS

Site characteristics

Site depths displayed minor variation amongst the five sampling regions. Depths were generally lowest for Tampa Bay, and highest for Anclote Keys (Table 1). Because of the shallow depths and relatively clear water, most regions displayed Secchi ratios near 1, and exhibited minor intra-regional variation. Regional comparisons reveal that water clarity was highest for Tampa Bay, Homosassa Bay, and Florida Bay. Anclote Keys and Charlotte Harbor displayed slightly reduced water clarity, whereby average Secchi depths

were 65% and 79% of the recorded site depth, respectively. Site temperatures were lowest for Homosassa Bay, and highest for Tampa Bay, while salinity was lowest in Charlotte Harbor and highest in Florida Bay.

Plant chemical characteristics

The nutrient and carbohydrate content of *Thalassia testudinum* displayed significant intra- and inter- regional variation for all sampling years (1997-1999). Across all regions, leaf nitrogen content (%N of dry mass) ranged from 1.60% to 3.96%, while leaf phosphorus content (%P of dry mass) ranged from 0.06% to 1.08%. Within each region, both leaf %N and %P were highest in Charlotte Harbor and lowest in Florida Bay (Table 1). Across all sites, carbon content displayed relatively little variation. Throughout the study, %P had higher coefficients of variation (0.16 - 0.26), as compared to %N (0.09 - 0.17) and %C (0.04 - 0.06). Regional variation in leaf N and P content produced marked variation in seagrass N:P ratios, which were lowest in Charlotte Harbor and highest in Florida Bay.

Rhizome carbohydrate content additionally displayed considerable variation across all sampling years (Table1). Carbohydrate content was highest for Homosassa Bay and lowest for Anclote Keys. Within each region, carbohydrate content displayed higher coefficients of variation (0.31-0.71) relative to nutrient content (0.09-0.26).

Relationships between plant nutrient and carbohydrate content

All sampled regions displayed significant negative correlations between leaf nutrient content (%N and %P) and rhizome carbohydrate content. Intra-regional correlations (Spearman's rank) between nutrient and carbohydrate content were strongest

for Charlotte Harbor (ρ=-0.73, $p<0.01$) and weakest for Tampa Bay (ρ=-0.28, $p<0.01$). Intermediate correlations were displayed for Homosassa Bay (ρ =-0.53, $p<0.01$), Anclote Keys (ρ =-.66, $p<0.01$), and Florida Bay (ρ =-0.46, $p<0.01$).Within each site, correlations were generally strongest for the nutrient which was in least supply for that respective region. Thus, regions with seagrass N:P ratios below 30 demonstrated higher correlation coefficients with %N as opposed to %P (with the exception of Anclote Keys). Conversely, regions with seagrass N:P ratios above 30 demonstrated highest correlations with %P. Linear regressions between carbohydrate and nutrient content revealed similar negative relationships for all regions (Fig. 1).

DISCUSSION

Intra- and inter-regional variation in the leaf nutrient content of *T. testudinum* was detected during our sampling period, as previously documented in prior studies for seagrasses within these regions (Fourqurean and Cai 2001). Among-region variation in leaf nutrient content was larger than within-region variation, generating significant differences in the nutrient content of *T. testudinum* across broad spatial scales. For example, leaf N:P ratios were low in Charlotte Harbor, indicating low nitrogen relative to phosphorus content. Conversely, Florida Bay displayed high N:P ratios, indicating high nitrogen relative to phosphorus content. These large scale changes may be due to a number of attributes. Previous work has documented shifts in the nutrient content of benthic plants, attributable to variation in the environmental availability and/or supply rates of nitrogen and phosphorus (Fourqurean et al. 1992a; Fourqurean and Zieman 2002). We suggest that similar variation in nutrient supply may be responsible for our

observed shifts in plant nutrient content. Changes in nutrient supply can result from either changes in nutrient loading rates and/or shifts in a number of abiotic factors (i.e., sediment mineralogy, sediment grain size, water clarity, or water depth) (Fourqurean and Zieman 2002). Such factors likely contributed to variation in plant nutrient content at both local and broad spatial scales.

Relationships between the leaf nutrient and rhizome carbohydrate content of the seagrass *T. testudinum* were detected during the course of a 3 year sampling period in Florida. While the strength of these relationships displayed regional variation, all sampling sites demonstrated significant negative correlations between leaf nutrient content (%N and %P) and rhizome carbohydrate content within each region. Here, we hypothesize that within each region, nutrient content may have influenced the carbohydrate storage reserves of this benthic plant because of the process of reserve accumulation that has been described in terrestrial plants (Chapin et al. 1990) . While the role of light availability certainly must be considered, we argue that the relatively consistent depths and Secchi values within each region suggest that these nutrient-carbohydrate relationships are not driven by large gradients in light availability. Terrestrial studies have documented that nutrient-limited plants are constrained in their ability to produce new biomass, thus carbon gain exceeds carbon demand, promoting the accumulation of storage compounds (Chapin 1980; Chapin et al. 1990; Wyka 2000). On the basis of our observations, we hypothesize that a similar mechanism may operate for *T. testudinum*. Prior work with the freshwater macrophyte, *Berula erecta* has experimentally demonstrated increased carbohydrate storage with nutrient limitation, and decreased carbohydrate storage under nutrient replete conditions (Puijalon et al. 2008).

Our field observations for *T. testudinum* follow similar trends, and we suggest that these preliminary observations warrant further manipulative experimentation.

The strength of these nutrient-carbohydrate relationships displayed inter-regional variation, as Spearman's rank correlations were highest for the Charlotte Harbor region, and lowest for the Tampa Bay region. Such variation may be attributable to large scale, regional differences in a number of abiotic factors (i.e., light, temperature, and/or salinity), which may regulate the strength of the nutrient-carbohydrate relationship. Previous work has demonstrated a number of factors which can influence seagrass carbohydrate content: for example season (Vichkovitten et al. 2007), light availability (Burke et al. 1996; Zimmerman and Alberte 1996; Lee and Dunton 1997; Carlson et al. 2003; Collier et al. 2009), and grazing (Fourqurean et al. 2010a). Large-scale regional variation in these factors may explain why the nutrient-carbohydrate relationships were relatively strong in some regions, while relatively weak in others.

The consistency of these negative relationships between nutrient and carbohydrate content within several geographically distinct regions suggests that seagrasses, such as *T. testudinum* with substantial storage organs, may accumulate reserves when some factor other than rates of photosynthetic carbon fixation limit plant biomass. We suggest that nutrient availability may need to be incorporated into the framework of factors that regulate the carbohydrate reserves of *T. testudinum*, and provide additional insight towards the storage dynamics of this benthic plant.

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Table 1: Abiotic measurements and seagrass leaf tissue chemistry (NSC = non-structural carbohydrate content) for each sampling region. Data from 1997-1999 were pooled. Values are means ± S.E.

Region	Depth (m)	Secchi Ratio	Temperature (°C)	Salinity	NSC (mg g^{-1} FM)	%C (% dry mass)	%N (% dry mass)	% P (% dry mass)	C:N	C:P	N:P
Homosassa Bay	1.66 ± 0.04	1.00 ± 0.01	25.5 ± 6.6	25.7 ± 4.8	40.7 ± 1.7	36.9 ± 0.2	2.39 ± 0.04	0.15 ± 0.01	18.3 ± 0.2	679.4 ± 12.3	37.5 ± 0.8
Anclote Keys	2.16 ± 0.06	0.65 ± 0.03	28.5 ± 3.8	29.7 ± 2.8	21.8 ± 1.6	35.7 ± 0.2	2.52 ± 0.03	0.21 ± 0.01	16.6 ± 0.2	466.1 ± 12.1	27.9 ± 0.6
Tampa Bay	1.07 ± 0.04	0.98 ± 0.01	30.6 ± 1.2	32.6 ± 0.8	38.9 ± 1.0	34.6 ± 0.2	2.46 ± 0.02	0.25 ± 0.01	16.6 ± 0.2	365.7 ± 5.1	22.2 ± 0.3
Charlotte Harbor	1.35 ± 0.06	0.79 ± 0.02	29.6 ± 2.4	17.0 ± 4.1	26.9 ± 1.9	37.9 ± 0.2	3.02 ± 0.04	0.70 ± 0.02	14.8 ± 0.2	148.4 ± 4.2	10.0 ± 0.2
Florida Bay	1.88 ± 0.07	1.00 ± 0.01	29.2 ± 2.1	36.7 ± 1.4	32.9 ± 1.2	35.7 ± 0.2	2.14 ± 0.02	0.09 ± 0.01	19.8 ± 0.2	1066.1 ± 23.3	53.5 ± 0.9

FIGURE CAPTIONS

Figure 1. Relationship between rhizome non-structural carbohydrate content (NSC) and leaf nutrient content (%N or %P) for each region from 1997-1999. Lines represent significant linear regressions. Mean elemental ratios for each region are indicated.

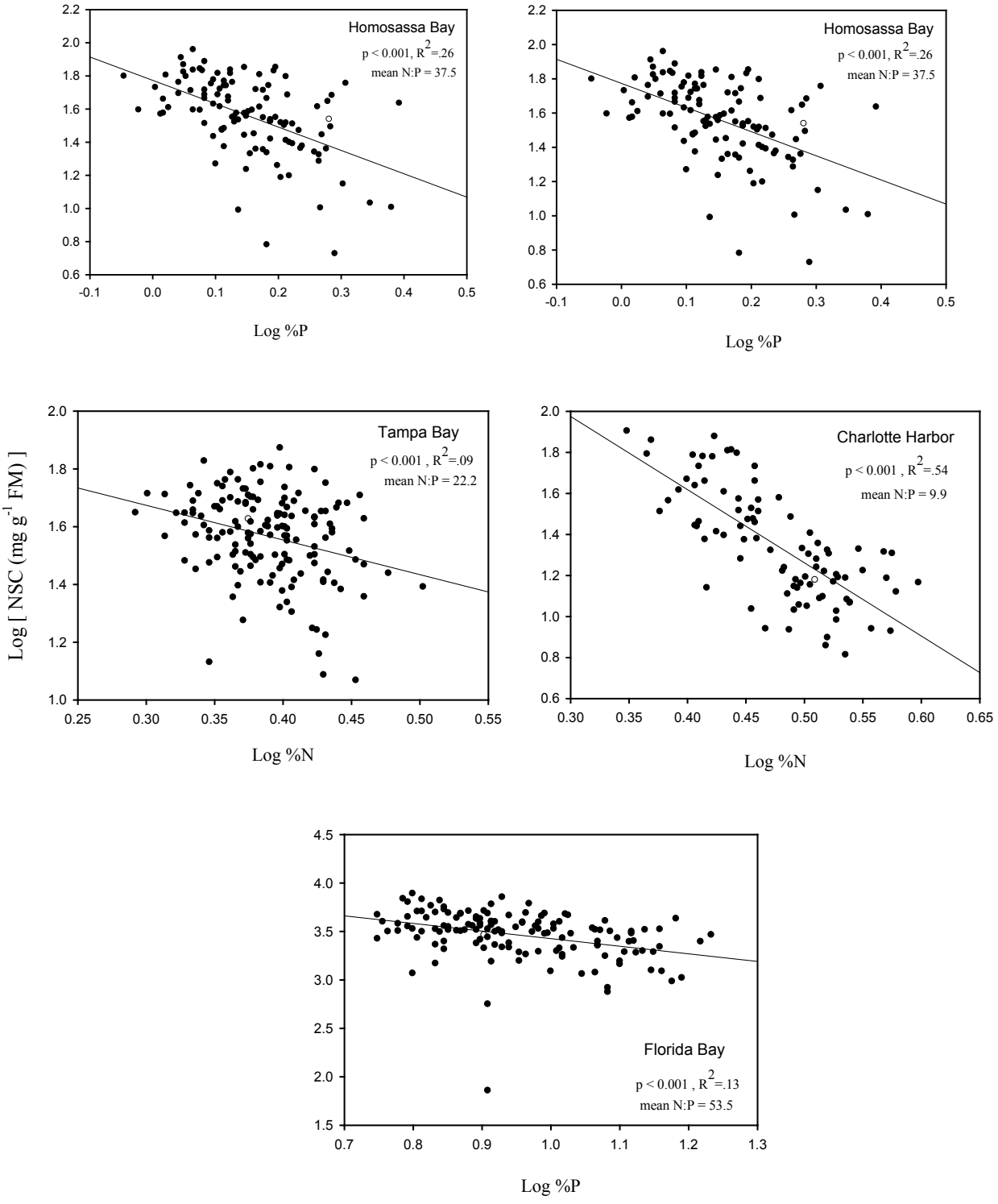
Homosassa Bay
p < 0.001, R2=.26
mean N:P = 37.5
Log %P
Homosassa Bay
p < 0.001, R2=.26
mean N:P = 37.5
Log %P
Log [NSC (mg g-1 FM)]
Tampa Bay
p < 0.001 , R2=.09
mean N:P = 22.2
Log %N
Charlotte Harbor
p < 0.001 , R2=.54
mean N:P = 9.9
Log %N
Florida Bay
p < 0.001 , R2=.13
mean N:P = 53.5
Log %P

FIGURE 1.

CHAPTER VI

LONG-TERM CARBON LIMITATION OF A NUTRIENT REPLETE SEAGRASS BED: A MANIPULATIVE FIELD EXPERIMENT

ABSTRACT

Climate change and future increases in oceanic $CO_{2(aq)}$ concentrations may provide a benefit to submerged plants by alleviating photosynthetic carbon limitation. However, we know relatively little in how this small scale benefit might translate into larger scale impacts beyond the performance of individual leaves. Similar to terrestrial research, these widely distributed ecosystems have the potential to undergo a number of functional changes in biomass production, nutrient cycling, and carbon storage with ongoing increases in $CO_{2(aq)}$ concentrations. To date, these long-term, large-scale responses have received limited attention. Furthermore, a number of alternate environmental factors (i.e., mineral resources) may serve to influence how seagrasses respond to additional $CO_{2(aq)}$ supply, potentially producing a number of complex interactions. To address these issues, this study manipulated both $CO_{2(aq)}$ and nutrient availability within a natural seagrass bed, and examined how long term *in situ* responses (productivity, biomass allocation, nutrient status, and sediment carbon storage) are influenced by nutrient availability. Clear open top chambers were used to replicate $CO_{2(aq)}$ forecast for the year 2100, while nutrient availability was manipulated via monthly additions of both nitrogen (N) and phosphorus (P) granular fertilizer. After 11 months, elevated nutrient availability increased plant N content, however there was no effect of nutrient addition on seagrass productivity, biomass allocation, or sediment carbon storage. Enrichment with $CO_{2(aq)}$ significantly increased total seagrass biomass and shoot:root ratios, yet had no effect on nutrient content or sediment carbon storage. Interactions between carbon and nutrient enrichment were not detected, suggesting that

nutrient availability did not influence seagrass response to additional $CO_{2(aq)}$. Thus, the nutrient replete status of our seagrass bed only revealed long-term carbon limitation in biomass production. Our study demonstrates that long-term alleviation of carbon limitation can increase biomass production of nutrient replete seagrass beds.

INTRODUCTION

Coastal regions will likely undergo a number of physiochemical changes associated with a series of climate change stressors. Because of close proximity to the air-sea interface, increasing concentrations of atmospheric CO_2 will predominantly influence shallow submerged environments, causing increases in $CO_{2(aq)}$ concentrations and declines in CO_3^{2-} concentrations. While negative impacts on a variety of calcified invertebrates are expected, a growing body of research has identified additional responses from a broad group of marine plants (seagrasses), which form a substantial component of benthic coastal systems around the world. Unlike calcified organisms, seagrasses may benefit from forecasted increases in $CO_{2(aq)}$ concentrations, which have been projected to nearly triple by the end of the century (Brewer 1997). While short-term physiological research has demonstrated photosynthetic gains for a large number of seagrasses in $CO_{2(aq)}$ rich environments (Durako 1993; Zimmerman et al. 1997), long-term ecological responses are still being evaluated. Comparatively, terrestrial plant CO_2 research has largely advanced due to the initiation of experiments which examine the impacts of multiple stressors on the CO_2 response of terrestrial systems under natural environments. Free Air Carbon Enrichment (FACE) experimentation has provided a window into how higher ecological properties change as a function of CO_2 enrichment. Because of

similarities in small-scale photosynthetic responses, many marine plants exposed to increasing $CO_{2(aq)}$ concentrations will likely undergo a number of similar ecological changes. Such similarities, present a strong case for drawing parallels between prior terrestrial ecosystem research and current seagrass $CO_{2(aq)}$ research, both in interpreting current findings, and directing future work.

Terrestrial ecosystem climate change research centers around the CO_2 fertilization effect, whereby vegetation responds to increases in CO_2 supply via elevated rates of carbon fixation and net primary production (Long et al. 2004). While small-scale, physiological plant responses to increased CO_2 are well established; large-scale ecosystem responses are increasingly nuanced, and may strongly depend upon the availability of alternate resources (Oren et al. 2001; Poorter and Perez-Soba 2001; Hungate et al. 2003; Luo et al. 2004; Reich et al. 2006; Norby et al. 2010). For example, because of the nutritional balance of plant vegetation, CO_2-stimulated production of additional biomass requires an increase in the supply rate of mineral resources (Rastetter et al. 1997). Thus, theory suggests that plant ecosystem responses to atmospheric CO_2 enrichment may become rapidly constrained by nutrient availability, and influenced by a variety of feedbacks (Rastetter et al. 1997; Luo et al. 2004). Nutrient-limited systems have shown show relatively small responses to CO_2 enrichment (Oren et al. 2001), while other systems have displayed shifts in plant nutrient content under CO_2 enrichment (Cotrufo et al. 1998; Norby et al. 2010).While these phenomena have been extensively explored within terrestrial systems, these carbon-nutrient interactions are relatively unexplored for marine plant communities in the context of future climate change.

Mesocosm and *in situ* field research has demonstrated a number of seagrass responses to $CO_{2(aq)}$ enrichment. Present day $CO_{2(aq)}$ concentrations are relatively low in seawater, thus laboratory and mesocosm research has documented carbon-limited photosynthetic rates for a large number of seagrass taxa (Durako 1993; Beer and Koch 1996; Invers et al. 1997; Zimmerman et al. 1997; Invers et al. 2001). Alterations in biomass partitioning and increases in non-structural carbohydrates (NSC) have been additionally noted in prior mesocosm research (Palacios and Zimmerman 2007; Jiang et al. 2010) and field $CO_{2(aq)}$ manipulations (Campbell and Fourqurean, in review). Increases in shoot densities have also been detected for seagrasses growing adjacent to volcanic CO_2 vents. While $CO_{2(aq)}$ mediated increases in production have received experimental support, few studies have attempted to examine how alternate resources (such as nutrient availability) might interact and influence seagrass responses to additional carbon supply.

Shifts in seagrass nutrient content with $CO_{2(aq)}$ enrichment have been documented in a limited number of studies. While this topic has received extensive attention in the terrestrial literature, many seagrass studies fail to consider the role that nutrient availability plays in influencing responses to CO_2 enrichment. Short-term, laboratory work by Jiang et al. 2010 report declines in leaf nutrient content under $CO_{2(aq)}$ enrichment for the tropical seagrass *Thalassia hemprichii*. *In situ* manipulations of $CO_{2(aq)}$ have similarly demonstrated declines in leaf nutrient for another tropical species, *Thalassia testudinum* (Campbell and Fourqurean, in review). While it has been proposed that non-structural carbohydrates (NSC) dilution is responsible for these declines in nutrient supply, the full mechanisms behind this phenomenon have yet to be detailed. Prior

terrestrial research has demonstrated that $CO_{2(aq)}$ mediated declines in plant nutrient content might only be partially attributable to NSC dilution (Tissue et al. 1997; Stitt and Krapp 1999), with other factors, such as the inability of nutrient uptake to meet CO_2 mediated increases in N and P demand playing a strong role. Because a large number of terrestrial studies document reduced or limited responses of CO_2 enrichment in nutrient - limited systems (Stitt and Krapp 1999), current seagrass research might be missing critical information on carbon-nutrient interactions in regards to $CO_{2(aq)}$ enrichment. The production of seagrass leaf material with elevated leaf carbon content, relative to nitrogen or phosphorus content can have implications for the long-term, CO_2 responsiveness of these systems. As seagrasses commonly inhabit environments of varying nutrient availability (Duarte 1990), it remains important to evaluate the impacts of $CO_{2(aq)}$ enrichment across a broad range of environmental conditions, and highlight the influence of other resources which regulate biomass production in the context of CO_2 enrichment.

The objective of this study was to assess the influence of nutrient availability on the responses of the seagrass *Thalassia testudinum* to field $CO_{2(aq)}$ manipulation. A novel technique of *in situ* carbon enrichment was used to mimic $CO_{2(aq)}$ concentrations forecast for the year 2100 around a shallow, naturally-occurring seagrass bed. Both $CO_{2(aq)}$ and nutrient (N and P) availability was manipulated for 11 months, while seagrass productivity and nutrient content was continuously monitored. Large-scale assessments of above / belowground biomass, shoot demographics, sediment carbon content, and non-structural carbohydrate content were additionally examined to provide an increasing comprehensive picture of ecosystem level responses.

METHODS

Study site

In situ manipulation of $CO_{2(aq)}$ concentrations and nutrient availability was conducted within a shallow (1m depth), nearshore (10m off-shore) seagrass bed in the Florida Keys, Florida, USA (24.55 N, 81.75 W). The benthic community was dominated by the seagrass *Thalassia testudinum*, with sparse abundances of the seagrasses *Syringodium filiforme* and *Halodule wrightii*. Several species of calcareous green algal species (*Halimeda* spp. and *Penicillus* spp.) were additionally present with a patchy distribution. The sediments were composed of roughly 9% organic matter; with the remaining mineral fraction consisting of fine biogenic calcium carbonates. Seawater temperatures were recorded every 6 hrs with a HOBO temperature logger. Light levels at the top of the seagrass canopy level within the chambers and open plots were assessed on a seasonal basis with 2pi PPFD sensor (WALZ, Diving PAM).

Experimental design

A balanced, 3 x 2 factorial experiment was designed to study the interactive effects of $CO_{2(aq)}$ and nutrient enrichment on seagrass epiphytes. The carbon treatment consisted of 3 levels ($CO_{2(aq)}$ enriched plots, $CO_{2(aq)}$ ambient plots, and controls), and the nutrient treatment consisted of 2 levels (nitrogen and phosphorus addition, +NP; and control, (C)). Thirty experimental seagrass plots were arranged in a randomized grid design (5 rows x 6 columns). Replicates (n=5) for each treatment were then randomly assigned within each column (Fig. 1). Seagrass plots (.17m^2) were spaced at 1 m intervals throughout the grid.

$CO_{2(aq)}$ enrichment

Optically-clear, open-top acrylic chambers were used to establish the carbon enriched seagrass plots (see Campbell and Fourqurean 2011 for a detailed description). Seawater enriched in $CO_{2(aq)}$ was generated in the field by bubbling pure CO_2 gas into a series of submerged water pumps, which subsequently delivered $CO_{2(aq)}$ enriched seawater into the carbon enriched chambers via an underwater PVC network. This technique has been demonstrated as an effective means of confining $CO_{2(aq)}$ enrichment to a localized area of the benthos. As carbon enriched seawater is pumped into the chambers, it cycles within the seagrass canopy before being flushed out the top. This design allows for long-term constraint of carbonate parameters within the enriched chambers while limiting the impacts of reduced light and water motion. The $CO_{2(aq)}$ ambient chambers received unenriched seawater from an independent PVC network connected to a separate series of water pumps. Open seagrass control plots lacked chambers, and were designated with a PVC frame. The level of $CO_{2(aq)}$ enrichment was carefully controlled, and set to mimic forecasts for the year 2100, roughly a 0.3 unit reduction in pH (Caldeira and Wickett 2003). On a weekly basis, all chambers were scrubbed clear of any fouling, and pH measurements (NBS scale, relative accuracy ± 0.002) were taken within all chambers and control plots during the 1200-1500 time period. Water samples were additionally collected periodically during the experiment to monitor salinity and total alkalinity. During each sampling, 20ml of seawater was sampled within each chamber, filtered through a 0.7 um GFF filter, and stored on ice until further processing. Total alkalinity was measured in the lab via automated, potentiometric titration with 0.1 N HCL, and salinity was measured with an Orion

conductivity meter. Carbonate parameters ($CO_{2(aq)}$, HCO_3, CO_3, and calcite/aragonite saturation states) were calculated with the CO_2Sys Excel Macro (Lewis and Wallace 1998), using the dissociation constants of Mehrbach et al (1973), refit by Dickson and Millero (1987).

Nutrient enrichment

Fertilizer was evenly distributed by hand over each designated +NP chamber or plot on a monthly basis. Nitrogen was added in the form of urea-coated slow-release N fertilizer (Polyon, Pursell Technologies; 38-0-0, 94% nitrogen as urea) and phosphorus was added as deflourinated granular phosphate rock [Multifos, IMC Phosphates] $Ca_3(PO_4)^2$, 18%P). Previous studies have effectively used these enrichment methods to increase nutrient availability to both the above- and belowground biomass of benthic macrophytes in South Florida (Ferdie and Fourqurean 2004). Final loading rates were 1.54 g N m^{-2} d^{-1} and 0.24 g P m^{-2} d^{-1}, and were similar to prior eutrophication studies within this region (Armitage et al. 2005; Armitage et al. 2011).

Seagrass productivity

Rates of seagrass leaf production were measured 6 times during the course of the 11 month enrichment period. Measuring leaf production requires the removal of multiple shoots, thus the number of sampling events was limited to prevent overharvesting within each chamber and open plot. During each productivity sampling, 3-5 shoots of *T. testudinum* within each chamber and open plot were pierced with a hypodermic needle near the base. These marked shoots were then harvested after 7-10 days of *in situ* growth. In the lab, epiphytes were removed with a razor, and the lengths and widths of all leaves

were recorded. Each shoot was divided into new and old leaf material, and dried to a constant weight in a 70°C oven. Relative leaf growth rates (mg new leaf mass * g^{-1} total leaf mass *day^{-1}), absolute leaf growth rates (mg new leaf mass*$shoot^{-1}$*day^{-1}) and total shoot mass (dry g*$shoot^{-1}$) for each shoot were calculated.

Seagrass nutrient content

Nutrient content of seagrass leaf material was measured in each chamber and control plot on the same sampling schedule as productivity measurements. During each of the 6 productivity sampling events, newly produced leaf material (leaf rank 1) from 3-5 shoots was analyzed for carbon (C), nitrogen (N), and phosphorus (P) content (% g dry wt). Dried leaf material was ground into a fine powder with a mortar and pestle, and analyzed for C and N content using a CHN analyzer. P content of dried leaf material was determined via dry oxidation, acid hydrolysis extraction followed by colorimetric analysis (Fourqurean et al. 1992a).

Sediment cores

Sediment cores were taken at the conclusion of the experiment to assess total organic content, organic carbon content, and inorganic carbon content. A single sediment core was taken within each replicate chamber and open plot with a 60 ml syringe (capturing the upper 10 cm of sediment) and dried to a constant weight in a 70°C oven. Sediment samples were passed through a (1mm) sieve to remove large particles, homogenized with a mortar and pestle, and aliquots were ashed at 500°C for 5h to determine total organic content (calculated as % wt loss on ignition). Ashed samples were further analyzed for inorganic carbon content (IC) using a CHN analyzer (Fisons

NA1500). Additional aliquots of the pre-ashed sediment samples were analyzed for total carbon content (TC) using a CHN analyzer, and organic carbon content (OC) was calculated as the difference between TC and IC.

Biomass partitioning

Benthic cores were used to assess total seagrass biomass and seagrass biomass partitioning at the end of the study. After 11 months of carbon and nutrient enrichment, 2, 15 cm diameter cores were taken within each chamber and control plot. Cores were pushed into the sediment down to the underlying limestone bedrock and all aboveground and belowground biomass was collected. Both cores from within each plot were placed in a single mesh bag, rinsed free of sediment and transported to the lab in coolers. In the lab, all shoots were counted, measured for length, and separated from belowground rhizomes and root fractions. Belowground biomass was additionally separated into live and dead biomass. Biomass fractions were dried to a constant weight in 70°C ovens to obtain dry weights.

Shoot demographics

All shoots within the biomass cores from each chamber and open plot were aged using prior seagrass demographic reconstruction techniques (Duarte et al. 1994). The leaf emergence rate (LER, new leaves/shoot/day) throughout the experimental period was determined by counting the proportion of shoots which produced new leaves during each of the 6 productivity sampling events, and dividing this proportion by the time interval between shoot marking and harvest. Plastochron interval (PI, days between successive leaf emergence) was calculated as the reciprocal of LER (averaged for each sampling

period). Within each biomass core, shoots with connections to horizontal rhizome were selected for aging. For these shoots, lifetime leaf production was determined by counting vertical rhizome leaf scars and extant green leaves. Shoot age was calculated as the product of total leaf production and average site PI.

Soluble carbohydrates

Non structural carbohydrate content (NSC) of seagrass horizontal rhizomes was determined using the MBTH (3-methyl-2-benzothiazolinone hydrazome hydrochlorie) analysis method (Johnson et al. 1981; Pakulski and Benner 1992). All rhizome material collected from the biomass cores was ground into a fine powder with a Wiley-Mill and transferred into 20ml glass scintillation vials. Chemical analysis of aliquots of each sample involved a borohydride reduction of hydrolyzed monosaccharides to sugar alcohols, followed by periodate oxidation to formaldehyde, and colorimetric determination of formaldehyde by MBTH (Lee and Dunton 1997). NSC is reported as mg soluble carbohydrate $*g^{-1}$ DW. Total soluble carbohydrate content on an areal basis was calculated by multiplying sample carbohydrate concentration by total rhizome dry weight.

Statistical analyses

Two-way repeated measures ANOVA was used to analyze repeated measurements of seagrass leaf growth and nutrient content, with sampling date as the within-subjects factor and $CO_{2(aq)}$ and nutrient treatment as the between subjects factor. To avoid pseudoreplication, statistical analyses were conducted with the means of replicate subsamples from within each chamber or open plot. Biomass partitioning,

sediment characteristics, and soluble sucrose content collected at the end of the experiment were analyzed with a two-way ANOVA. All data were tested for normality and variance homogeneity, and when violated, data were either log transformed, or ranked and analyzed non-parametrically.

RESULTS

Enrichment with $CO_{2(aq)}$was effectively maintained within the enriched chambers throughout the 11 month experiment (Table 1). Seawater temperature and pH were measured a total of 35 times within each chamber and open control plot. The enriched chambers ($CO_{2(aq)}$, $CO_{2(aq)}$ + NP) displayed mean pH values (± 1SE) of 7.86 (± 0.03) and 7.90 (± 0.03) respectively. The unenriched chambers (No $CO_{2(aq)}$, No $CO_{2(aq)}$ NP) displayed mean pH values (± 1SE) of 8.20(± 0.02) and 8.19 (± 0.02) respectively, while the open control plots (Control , Control NP) both displayed pH values of 8.19 (± 0.02). The difference (mean ± 1SE) in pH between the $CO_{2(aq)}$ enriched and unenriched chambers and open plots was 0.32 ± 0.02 pH units. Calculated $CO_{2(aq)}$ parameters followed similar trends, with the enriched chambers ($CO_{2(aq)}$, $CO_{2(aq)}$+NP) displaying mean $CO_{2(aq)}$ concentrations (± 1 SE) of 29.9 ± 2.6 and 27.4 ± 2.6 umol / kg SW respectively. The ambient chambers (No $CO_{2(aq)}$, No $CO_{2(aq)}$ NP) displayed mean $CO_{2(aq)}$ concentrations of 11.3 ± 0.6 and 11.1 ± 0.6 respectively, while the control plots (Control , Control NP) averaged 11.6 ± 0.6 and 11.5 ± 0.6 respectively. Furthermore, bicarbonate (HCO_3^-) and total dissolved inorganic carbon (DIC) concentrations were significantly increased within the $CO_{2(aq)}$ enriched chambers as compared to the ambient chambers and open controls.

Within each of the 35 water sampling events, the $CO_{2(aq)}$ enriched chambers displayed higher pH variation among replicate chambers as compared to the unenriched chambers and open plots (Fig. 2). The $CO_{2(aq)}$ enriched treatment (+$CO_{2(aq)}$;+ $CO_{2(aq)}$/ +NP) displayed a mean pH variation among chambers of 4.1 and 3.1% respectively. Comparatively, both ambient chamber treatments (No $CO_{2(aq)}$; No $CO_{2(aq)}$ +NP) displayed mean pH chamber variation of 0.4%, while the open plots (Open ; Open +NP) displayed pH variation of 0.3 and 0.1% respectively. However, averaged over the course of the experiment, calculated 95% confidence intervals indicate that pH values were not statistically different between individual chambers within each treatment, suggesting that no replicate chamber or open plot displayed consistently abnormal pH values within each treatment. Repeated-measures ANOVA additionally revealed a significant time ($F = 40.5$, $P < 0.001$), and time x carbon interaction ($F = 8.3$, $P < 0.001$) for chamber pH values, indicating that the $CO_{2(aq)}$ enriched chambers responded differently to ambient seasonal variation in seawater carbonate parameters.

Total alkalinity (means ± 1SE) was 2508.9 ± 41.6 and was not impacted by $CO_{2(aq)}$ enrichment or the chamber structure. Salinity averaged 35.5 throughout the experiment, ranging from 33.9 - 37.8. Midday water temperatures averaged 29.4°C and ranged from 33.9°C in August to 19.6°C in December. There was no impact of the chamber structure itself on seawater temperatures. Prior work has demonstrated a slight (5%) reduction in light levels imposed by the chamber structures (Campbell and Fourqurean 2011), however light levels inside the chambers were 600-700 umol photons m^{-2} s^{-1} at the top of the seagrass canopy during the noon time period.

Nutrient fertilization increased seagrass nitrogen content (%DW), yet had no impact on phosphorus content (Table 2, Figure 3, Figure 4). Across carbon treatments, nitrogen content (means ± 1SE) was 2.28 ± 0.09% in the unfertilized chambers and open plots, and 2.47 ± 0.1% in the fertilized chambers and open plots. Fertilized seagrasses displayed lower C:N ratios (17.3± 0.7) and higher N:P ratios (38.1 ± 2.5) compared to unfertilized seagrasses (18.2 ± 0.7 and 35.3 ± 2.1, respectively). Carbon dioxide enrichment altered phosphorus content (%DW) and N:P ratios, however significant differences were only between the unchambered open plots and the enriched chambers. Mean seagrass leaf P content was 0.14 ± 0.01 % (± 1SE) within the $CO_{2(aq)}$ enriched chambers, and was 0.15 ± 0.01% and 0.16 ± 0.01% within the unenriched chambers and open controls. Mean seagrass leaf N:P ratios were 38.3 ± 2.2 within the $CO_{2(aq)}$ enriched chambers, and was 37.5 ± 2.3 and 34.2 ± 2.3 within the unenriched chambers and open plots. Leaf nitrogen content (%DW) was not altered by $CO_{2(aq)}$ treatment. The within subjects factor of time was significant for all nutrient response variables, and significant time x nutrient interactions were detected for leaf nitrogen content ($F = 4.24$, $P = 0.004$) and N:P ratio ($F = 4.415$, $P = 0.003$).

Seagrass leaf growth rates varied during the course of the experiment, and were significantly altered by nutrient enrichment (Table 2 Fig 5). Across carbon treatments, mean relative leaf growth rates were 22.4 ± 1.6 mg g^{-1} d^{-1} within the nutrient enriched chambers, and 21.2 ± 1.5 mg g^{-1} d^{-1} (± 1 SE) within the unenriched chambers and open controls. $CO_{2(aq)}$ enrichment had no effect on leaf growth rates. Seagrass shoot mass additionally varied during the course of the experiment, and was significantly altered by

carbon treatment with elevated shoot mass within the $CO_{2(aq)}$ ambient chambers as compared to the open controls (Table 3,4).

Seagrass cores at the end of the experiment revealed that carbon enrichment significantly increased total live biomass (AG+BG) within the $CO_{2(aq)}$ enriched chambers as compared to the ambient chambers ($F = 4.92$, $P = 0.017$), while nutrient enrichment had no effect on live biomass (Table 3,4; Fig 6). Across nutrient treatment, mean biomass within the $CO_{2(aq)}$ enriched chambers was 385.9 ± 39.2 ($\pm$ 1 SE) g m^{-2} and was 280.55 ± 61.3 g m^{-2} within the $CO_{2(aq)}$ ambient chambers. Mean biomass within the unchambered open plots (402.8 ± 16.3 g m^{-2}) was not significantly distinct from the $CO_{2(aq)}$ enriched chambers. Carbon treatment additionally altered seagrass shoot:root (S:R) ratios, however significant differences were only detected between the $CO_{2(aq)}$ enriched chambers and the unchambered control plots. Averaged across nutrient treatment, mean S:R ratios within the $CO_{2(aq)}$ enriched chambers were 1.1 ± 0.2 ($\pm$ 1SE), while ratios within the unenriched chambers and open plots were 0.76 ± 0.23 and 0.54 ± 0.1, respectively. Nutrient enrichment did not alter seagrass S:R ratios (Fig. 6). Furthermore, significant interactions between carbon and nutrient enrichment were not detected for either live biomass or S:R ratio. Seagrass standing crop, shoot density, or shoot age were not altered by either $CO_{2(aq)}$ or nutrient enrichment, however root biomass was altered by carbon treatment, with significantly higher values within the unchambered open plots as compared to ambient chambers. Lastly, neither sediment cores metrics (organic matter, organic carbon, and inorganic carbon) nor rhizome soluble carbohydrate content were significantly altered by $CO_{2(aq)}$ or nutrient enrichment (Table 3,4).

DISCUSSION

In situ $CO_{2(aq)}$ enrichment increased the total biomass of a tropical seagrass community over an 11 month enrichment period. These results are consistent with other seagrass $CO_{2(aq)}$ enrichment studies under both mesocosm (Palacios and Zimmerman 2007) and field conditions (Hall-Spencer et al. 2008), suggesting that prior assessments of increases in seagrass productivity from laboratory experiments may be realized with prolonged increases in coastal dissolved $CO_{2(aq)}$ concentrations (Beer and Koch 1996). While nutrient enrichment increased plant N content and relative leaf growth rates, interactions with carbon enrichment were not detected; thus, contrary to our hypothesis, nutrient availability did not influence seagrass response to carbon enrichment.

Long-term nutrient enrichment produced relatively minor effects on seagrass leaf nutrient content, relative leaf growth rates, or final biomass metrics; suggesting that factors other than nutrient availability likely control seagrass productivity at this site. The relatively dense leaf canopy at this site (400-700 shoots m^{-2}), and weak responses to nutrient enrichment suggests that light and space probably exert more proximate control on seagrass productivity as opposed to nutrient availability. While nutrient enrichment did increase leaf growth rates, the effect was relatively minor (mean increase of 5.5% over the controls), and did not result in significant increases in any final biomass metric measurements. Prior work in South Florida has demonstrated that seagrass beds towards the western limits of Florida Bay are relatively unresponsive to nutrient enrichment, displaying no responses in either leaf tissue chemistry or growth / biomass metrics (Armitage et al. 2005). These trends were explained by a relatively balanced supply of N

and P as indicated by seagrass leaf N:P ratios, which were near 30:1 (Atkinson and Smith 1983). Seagrasses at our site similarly displayed leaf N:P ratios near 30:1, thus further supporting the conclusion of balanced supply rates of nutrients to support seagrass production. Prior studies have suggested that seagrasses with N content above 1.8% are generally not limited by nitrogen supply, and seagrasses with P content above 0.2% are generally not limited by phosphorus supply (Duarte 1990). Mean seagrass nutrient content at our site was 2.37%N (supporting the nitrogen threshold), however P content was 0.15% DW (suggesting slight P limitation). While contradictory to this P threshold, other studies have documented no seagrass response to P enrichment despite relatively low tissue concentrations (≈ 0.10%P) (Ferdie and Fourqurean 2004), suggesting complex patterns of P limitation for primary producers in this region (Armitage et al. 2005). Despite minor growth responses to nutrient enrichment, seagrass leaf tissue responded to N addition by significantly increasing %N and N:P ratios, and reducing leaf C:N ratios. Such trends might indicate a degree of "luxury consumption" whereby nutrient uptake exceeds current growth demand (Chapin et al. 1990). Moreover, we find no long-term effect of $CO_{2(aq)}$ enrichment on seagrass nutrient content, contrasting prior work which documents $CO_{2(aq)}$ mediated declines in leaf N and P content (Campbell and Fourqurean, in review, Chapter 4) . While terrestrial studies widely document declines in plant N content with $CO_{2(aq)}$ enrichment (Cotrufo et al. 1998), this effect is variable and can depend upon local nutrient availability (Stitt and Krapp 1999). Thus, additional research will be required to further document the effects of $CO_{2(aq)}$ supply on seagrass nutrient content.

Seagrass biomass significantly increased with elevated $CO_{2(aq)}$, suggesting carbon limited biomass production over extended time periods. Thus, continuing increases in coastal $CO_{2(aq)}$ concentrations might serve to significantly increase seagrass biomass in areas that are not limited by mineral resources. Individual biomass metrics assessed towards the conclusion of the experiment (standing crop, shoot density, rhizome biomass, and root biomass) were not significantly altered by $CO_{2(aq)}$ enrichment, however total biomass (both aboveground and belowground components) was altered. Therefore, only the summation of both aboveground and belowground biomass metrics reveal the effects of $CO_{2(aq)}$ enrichment. However, note that chamber effects were present, as the unchambered open plots had more total seagrass biomass in comparison to the ambient chambers, potentially attributable to increases in belowground biomass within the open plots. Because total biomass is a composite metric, significant differences between $CO_{2(aq)}$ treatments might be due to a variety of factors. Thus, differences between the chambered treatments (enriched and ambient $CO_{2(aq)}$) might be because of shifts in aboveground biomass, while differences between the ambient chambers and the open control plots more attributable to shifts in belowground biomass. While insignificant, seagrass standing crop and shoot density suggest that aboveground biomass was slightly elevated within the $CO_{2(aq)}$ enriched chambers as compared to the ambient chambers, whereas many of the belowground metrics (root and rhizome biomass) were higher within the unchambered open plots as compared to the ambient chambers. These trends suggest that the factors driving differences in total biomass were distinct between comparisons for the chambered treatments and the open plots. Lastly, we find that shoot:root ratios were higher within the $CO_{2(aq)}$ enriched chambers as compared to the

unchambered open plots, suggesting that, in general, aboveground biomass was higher within the $CO_{2(aq)}$ enriched chambers and lower within the unchambered open plots.

The long-term increases in seagrass biomass with carbon enrichment supports results from prior long-term seagrass mesocosm and field research, and follows widespread documentation of $CO_{2(aq)}$ mediated increases in plant biomass for a number of terrestrial systems (Bazzaz 1990; Korner 2000; Long et al. 2004; Ainsworth and Long 2005). Similar to these terrestrial systems, we document long-term increases in seagrass biomass under $CO_{2(aq)}$ enrichment, potentially altering the future functionality of these systems. Palacios and Zimmerman (2007) similarly document increases in the biomass of the temperate seagrass *Zostera marina* under prolonged $CO_{2(aq)}$ enrichment, however these shifts were solely attributable to changes in belowground biomass. Results from our study hint that biomass increases resulted from shifts in aboveground biomass, thus highlighting varying plant responses to increased $CO_{2(aq)}$. Terrestrial studies have demonstrated varying aboveground and belowground responses of plants to $CO_{2(aq)}$ enrichment (Stitt and Krapp 1999), potentially attributable to the effect of environmental conditions and mineral resource availability. As a growth strategy, plants direct growth and vegetative expansion towards acquiring resources in least supply (Bloom et al. 1985). Thus, under elevated $CO_{2(aq)}$, nutrient-replete plants might invest in aboveground shoot proliferation, whereas nutrient-deplete plants might invest in belowground structures. We suggest that a similar mechanism might operate for seagrasses, and account for the disparity between this study and that of Palacios and Zimmerman (2007). Some terrestrial work has documented $CO_{2(aq)}$ mediated shifts in S:R ratios which depend upon resource availability, thus S:R ratios decline under nutrient deplete conditions, and increase under

nutrient replete conditions (Stitt and Krapp 1999). The increase in S:R ratios under nutrient-replete $CO_{2(aq)}$ enrichment documented in this study follows findings from prior terrestrial work (Baxter et al. 1997; Geiger et al. 1999). Other seagrass studies which document the long-term impacts of increased $CO_{2(aq)}$ from volcanic vents further document increases in aboveground shoot density as a response to prolonged $CO_{2(aq)}$ (Hall-Spencer et al. 2008). However, Hall-Spencer et al. (2008) only reports shifts in shoot densities (shoots m^{-2}), and not standing crop (g m^{-2}), thus limiting our ability to draw conclusions about total seagrass biomass on an areal basis. Given the disparity in seagrass aboveground and belowground responses to $CO_{2(aq)}$ enrichment, we suggest that future work will benefit from studies which manipulate a number of environmental conditions (such as mineral resource availability) along with $CO_{2(aq)}$ supply. Similar to other long term $CO_{2(aq)}$ manipulation experiments (Palacios and Zimmerman 2007, Campbell and Fourqurean, in review, Chapter 4), we document no growth responses on the scale of individual shoots. Mass specific leaf growth rates were not impacted by $CO_{2(aq)}$ enrichment, nor did this study detect any shifts in the leaf area of individual shoots. These trends further suggest that seagrass productivity responses to long term $CO_{2(aq)}$ enrichment may be poorly represented at the scale of individual shoots, suggesting the need to integrate productivity measurements beyond individual shoots (Palacios and Zimmerman 2007).

Long-term $CO_{2(aq)}$ enrichment had no effect on seagrass rhizome soluble carbohydrates, contrary to the results of prior research mesocosm and field (Zimmerman et al. 1997; Jiang et al. 2010; Campbell and Fourqurean in review). These disparities suggest that the impact of $CO_{2(aq)}$ enrichment on NSC production may be variable, and

the lack of a significant NSC response might be attributable to differences in experimental duration and the timing of biomass harvest. Previous work documenting increases in seagrass NSC with $CO_{2(aq)}$ enrichment were the result of either short-scale (several weeks) or intermediate-scale (several month) responses. The present study examines long-term impacts (≈ 1 year), thus $CO_{2(aq)}$ mediated responses in NSC content might be masked by the seasonal variation in seagrass carbohydrate pools (Lee and Dunton 1996). Because of the destructive nature of sampling rhizome carbohydrates, we limited our assessments to the end of the study to maintain the integrity of our seagrass plots. Thus, any gains in seagrass NSC content from $CO_{2(aq)}$ enrichment may have been eliminated by the investment of this additional resource towards alternate biomass compartments. Lee and Dunton (1996) observed that seagrasses with extensive rhizomes such as *T. testudinum*, can utilize stored NSC to fuel respiration during the winter season, or provide resources necessary for leaf growth during the spring. Our NSC sampling at the end of the study in July may have occurred after carbohydrate reallocation towards other biomass compartments. The $CO_{2(aq)}$ enriched chambers did display marginally significant increases in seagrass standing crop, thus any increase in NSC as shown in shorter term experiments, may have resulted in increased biomass over longer time periods. Terrestrial studies have demonstrated that plant soluble carbohydrates can be redirected towards a variety of biomass compartments (Chapin et al. 1990), and furthermore the effects of $CO_{2(aq)}$ enrichment on plant responses can vary according to harvest schedules and / or experimental time scales (Korner 2000). Thus, we suggest that NSC accumulation in $CO_{2(aq)}$ enriched seagrasses may represent a short-term response, while shifts in biomass compartments represent long-term trends. The only other long -

term (≈ 1 year) seagrass $CO_{2(aq)}$ enrichment study similarly fails to document significant increases in NSC at the end of experimentation (Palacios and Zimmerman 2007).

Nutrient or $CO_{2(aq)}$ enrichment did not have a significant impact on organic matter (OM) or organic carbon (OC) content. Similar to terrestrial systems, seagrass meadows are now being recognized as substantial hotspots for long term carbon (C) storage (Fourqurean et al. 2012), thus any $CO_{2(aq)}$ mediated increase in seagrass productivity might serve to promote C storage within these systems. While terrestrial work has demonstrated increases in belowground C storage with $CO_{2(aq)}$ enrichment, these trends are only evident over very long time scales, often taking more than four years before becoming statistically evident (Luo et al. 2006). Thus, this study may be been of insufficient duration to detect significant shifts in sediment fractions of organic carbon.

This study demonstrates that biomass production in nutrient replete seagrass beds may be carbon-limited over extended time periods. After an 11 month enrichment period, nutrient addition did not alter seagrass biomass; whereas $CO_{2(aq)}$ addition significantly increased total above- and belowground biomass. These results follow the conclusions of prior long-term research (Palacios and Zimmerman 2007; Hall-Spencer et al. 2008) and suggest that future increases in $CO_{2(aq)}$ may prove beneficial to a wide range of submerged plants. Compared to prior research, we observed a number of scale-dependent impacts of $CO_{2(aq)}$ enrichment, with short and intermediate scale responses being distinct from longer term responses. We suggest that future research needs to consider $CO_{2(aq)}$ enrichment responses in the context of experimental duration. Furthermore, we know relatively little about responses over a number of environmental gradients, thus studying seagrass $CO_{2(aq)}$ impacts under varying light, temperature, and nutrient regimes will

contribute to an increasingly comprehensive view of how future ocean acidification will alter these systems.

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Table 1. Seawater carbonate parameters observed within the chambered and open control plots. Values represent the averages of repeated measurements (1200-1500 time frame) taken during the course of the 11 month enrichment experiment. Bracketed values represent 95% confidence intervals. During the experiment total alkalinity averaged 2503 umol kg^{-1}, while temperature and salinity averaged 29.1° and 35.5 respectively.

Seawater parameter	Control NP	Control	No CO_2 NP	No CO_2	CO_2 NP	CO_2
pH (NBS scale)	8.19 (8.22-8.16)	8.19 (8.22-8.15)	8.19 (8.22-8.16)	8.20 (8.23-8.17)	7.90 (7.95-7.84)	7.86 (7.92-7.81)
DIC (umol/kg SW)	2140 (2190 - 2089)	2140 (2191-2090)	2136 (2185-2087)	2132 (2180-2083)	2305 (2360-2250)	2319 (2374-2263)
CO_2 (umol/kg SW)	11.5 (12.7-10.3)	11.6 (12.8-10.4)	11.3 (12.4-10.2)	11.1 (12.2-10.0)	27.4 (32.6-22.3)	29.9 (35.0-24.8)
HCO_3 (umol/kg SW)	1865 (1922-1808)	1866 (1924-1809)	1860 (1914-1805)	1853 (1907-1798)	2119 (2181-2057)	2138 (2200-2075)
CO_3 (umol/kg SW)	263 (280-246)	262 (280-245)	265 (281-249)	268 (284-252)	159 (176-141)	151 (169.6 - 132.6)
PCO_2 (uatm)	445.4 (488.7-402.0)	447 (490-404)	437 (476-398)	429 (468-390)	1054 (1243-864)	1147 (1329-965)
Ω Calcite	6.4 (6.8-6.0)	6.3 (6.8-5.9)	6.4 (6.8-6.0)	6.5 (6.9-6.1)	3.8 (4.3 - 3.4)	3.7 (4.1-3.2)
Ω Aragonite	4.3 (4.5-4.0)	4.2 (4.5-4.0)	4.3 (4.6-4.0)	4.3 (4.6-4.1)	2.6 (2.9-2.3)	2.4 (2.8-2.1)

Table 2. Results of 2-way repeated measures ANOVA on seagrass growth, shoot mass, leaf area, and nutrient content. Significant results are indicated in bold. Post hoc results (Holm-Sidak, $\alpha = 0.05$) are indicated.

	Relative leaf growth	Shoot mass	Leaf area	%C	%N
Source of variation					
within subjects factor					
time	**F = 106.9 , P < 0.001**	**F = 21.4 , P < 0.001**	**F = 21.1 , P < 0.001**	**F = 88.46 , P < 0.001**	**F = 103.7 , P < 0.001**
time x carbon	F = 0.981 , P = 0.453	F = 1.195 , P = 0.302	F = 1.871 , P = 0.176	F = 1.492 , P = 0.173	F = 1.034 , P = 0.481
time x nutrient	F = 0.380 , P = 0.804	F = 0.229 , P = 0.949	F = 0.060 , P = 0.808	F = 1.746 , P = 0.148	**F = 4.237 , P = 0.004**
time x carbon x nutrient	F = 0.641 , P = 0.726	F = 0.803 , P = 0.626	F = 0.260 , P=0.774	F = 1.942 , P = 0.065	F = 2.000 , P = 0.057
between subjects factor					
carbon	F = 3.029 , P = 0.07	**F = 5.758 , P = 0.01**	F = 2.466 , P = 0.106	F = 2.653 , P = 0.095	F = 0.103 , P = 0.903
posthoc results		($No\ CO_2$ > Open)			
nutrient	**F = 8.889 , P = 0.007**	F = 0.026 , P = 0.873	F= 0.215 , P = 0.647	F = 1.481 , P = 0.238	**F = 24.43 , P < 0.001**
posthoc results	(+NP > -NP)				(+NP > -NP)
carbon x nutrient	F = 1.862 , P = 0.180	F = 2.011 , P = 0.159	F = 0.523 , P = 0.599	F = 0.472 , P = 0.631	F = 1.273 , P = 0.302
posthoc results					

	% P	C:N	C:P	N:P
Source of variation				
within subjects factor				
time	**F = 36.5 , P < 0.001**	**F = 52.92 , P < 0.001**	**F = 20.21 , P < 0.001**	**F = 9.026 , P < 0.001**
time x carbon	F = 1.568 , P = 0.149	F = 0.817 , P = 0.590	F = 1.002 , P = 0.435	F = 1.551 , P = 0.154
time x nutrient	F = 1.285 , P = 0.283	F = 1.846 , P = 0.128	F = 2.411 , P = 0.072	**F = 4.415 , P = 0.003**
time x carbon x nutrient	F = 1.289 , P = 0.262	F = 1.134 , P = 0.350	F = 2.032 , P = 0.071	F = 1.353 , P = 0.231
between subjects factor				
carbon	**F = 4.248 , P = 0.03**	F = 1.206 , P = 0.320	F = 3.428 , P = 0.054	**F = 4.516 , P = 0.025**
posthoc results	(Open > CO_2)			(CO_2 > Open)
nutrient	F = 0.000 , P = 0.983	**F = 7.437 , P = 0.013**	F = 0.000 , P = 0.999	**F = 8.319 , P = 0.01**
posthoc results		(-NP > +NP)		(+NP > -NP)
carbon x nutrient	F = 0.283 , P = 0.757	F = 0.486 , P = 0.622	F = 1.646 , p = 0.219	F = 2.832 , P = 0.084
posthoc results				

Table 3. Results of biomass metrics (means ± 1SE) from seagrass cores. Significant differences are indicated in bold.

Biomass metric	CO_2	No CO_2	Open	CO_2 NP	NO CO_2 NP	Open NP
Standing crop (g DW m^{-2})	77.7 ± 15.1	48.3 ± 15.0	62.8 ± 4.4	81.0 ± 10.3	49.6 ± 17.8	51.5 ± 6.2
Shoot density (shoots m^{-2})	701.8 ± 131.6	438.6 ± 119.8	537.3 ± 25.4	657.9 ± 34.7	493.4 ± 150.1	466.0 ± 79.1
Shoot age (years)	2.34 ± 0.5	1.13 ± 0.2	1.86 ± 0.4	1.56 ± 0.2	1.84 ± 0.2	2.36 ± 0.4
Live rhizome (g DW m^{-2})	159.5 ± 30.9	137.0 ± 26.2	175.0 ± 28.1	217.5 ± 24.6	145.3 ± 34.2	172.1 ± 40.3
Total Rhizome (g DW m^{-2})	243.3 ± 31.7	178.9 ± 22.5	217.0 ± 16.4	288.0 ± 46.6	197.8 ± 33.2	270.2 ± 55.6
Active meristems (# m^{-2})	153.5 ± 33.1	82.2 ± 17.3	109.6 ± 30.0	153.5 ± 41.2	89.1 ± 30.4	95.9 ± 26.2
Live roots (g DW m^{-2})	94.5 ± 20.4	61.3 ± 4.1	120.7 ± 16.4	74.5 ± 16.5	55.8 ± 11.1	113.4 ± 40.6
Total Roots (g DW m^{-2})	182.9 ± 29.6	130.7 ± 13.7	198.2 ± 11.7	165.4 ± 32.1	134.0 ± 29.2	280.4 ± 46.2
Live BG biomass (g DW m^{-2})	254.0 ± 24.6	198.3 ± 29.0	295.7 ± 20.6	291.9 ± 37.4	201.2 ± 36.0	285.5 ± 43.0
Total BG biomass (g DW m^{-2})	426.3 ± 56.2	309.6 ± 27.1	415.2 ± 24.3	453.4 ± 76.1	331.8 ± 54.4	550.7 ± 90.5
Live biomass (AG + BG) (g DW m^{-2})	**360.5 ± 40.7**	**277.0 ±48.9**	**392.9 ± 20.0**	**411.3 ± 37.7**	**284.1 ± 73.6**	**412.7 ± 12.6**
Total biomass (AG +BG) (g DW m^{-2})	532.7 ± 66.4	388.2 ± 40.9	512.4 ± 25.5	572.7 ± 78.1	411.9 ± 91.7	671.6 ± 104.9
live AG:live BG biomass	0.41 ± .04	0.37 ± .08	.34 ± .04	0.44 ± .08	0.36 ± .06	0.26 ± .02
AG:BG biomass	0.25 ± .04	0.25 ± .07	.23 ± .02	.29 ±.05	.21 ± .04	.15 ± .03
Live Shoot:Root	**0.91 ± 0.2**	**0.76 ± 0.2**	**0.59 ± .07**	**1.32 ± 0.29**	**0.75 ± 0.26**	**0.49 ± 0.13**
Soluble carbohydrates (mg C g^{-1})	148.6 ± 5.6	147.3 ± 4.6	135.4 ± 5.0	150.0 ± 6.4	150.0 ± 4.0	149.7 ± 7.2
Areal soluble carbohydrates (mg C m^{-2})	765.9 ± 183.5	739.6 ± 148.6	855.9 ± 130.7	1199.5 ± 165.7	806.1 ± 193.4	905.6 ± 187.6
Sediment OM (% DW)	0.09 ± .008	0.08 ± 0.008	0.10 ± 0.009	0.09 ± .007	0.09 ± .005	0.10 ± .007
Sediment OC (% DW)	3.51 ± .33	2.95 ± .33	3.87 ± 0.42	3.36 ± .31	3.68 ± .29	3.68 ± 0.37
Sediment IC (% DW)	9.41 ± .24	10.0 ± .21	9.59 ± .24	9.71 ± 0.16	9.64 ± .12	9.31 ± 0.16

Table 4. Results of 2-way ANOVA on final seagrass biomass metrics. Significant differences are indicated in bold. Post hoc results were determined by a Holm-Sidak test ($\alpha = 0.05$)

	Source of Variation			Carbon Posthoc
	Carbon	Nutrients	Carbon x Nutrients	
Standing crop	F = 3.291 , P = 0.056	F = 0.0478 , P = 0.829	F = 0.200 , P = 0.820	
Shoot density	F = 2.722 , P = 0.088	F = 0.0606 , P = 0.808	F = 0.213 , P = 0.810	
shoot age	F = 1.663 , P = 0.214	F = 1.013 , P = 0.326	F = 3.146 , P = 0.064	
Live rhizome	F = 1.411 , P = 0.263	F = 0.640 , P = 0.431	F = 0.464 , P = 0.635	
Total Rhizome	F = 2.450 , P = 0.108	F = 1.580 , P = 0.221	F = 0.144 , P = 0.867	
Active meristems	F = 2.636 , P = 0.094	F = 0.08 , P =0.930	F = 0.054 , P = 0.947	
Live roots	**F = 3.748 , P = 0.038**	F = 0.394 , P = 0.536	F = 0.691 , P = 0.933	Open > No CO_2
Total Roots	**F = 7.825 , P = 0.002**	F = 0.462 , P = 0.503	F = 1.869 , P = 0.176	Open > No CO_2
Live belowground biomass	**F = 4.346 , P = 0.024**	F = 0.145 , P = 0.706	F = 0.290 , P = 0.751	Open > No CO_2
Total belowground biomass	**F = 4.563 , P = 0.021**	F = 1.234 , P = 0.278	F = 0.719 , P = 0.497	Open > No CO_2
Live biomass	**F = 4.921 , P = 0.017**	F = 0.872 , P = 0.361	F = 0.372 , P = 0.694	CO_2 , Open > No CO_2
Total biomass	**F = 4.142 , P = 0.03**	F = 1.715 , P = 0.204	F = 0.553 , P = 0.583	Open > No CO_2
Live AG:live BG biomass	F = 2.207 , P = 0.134	F = 0.152 , P = 0.701	F = 0.411 , P = 0.668	
AG:BG biomass	F = 1.364 , P = 0.276	F = 0.477 , P = 0.497	F = 0.800 , P = 0.462	
Shoot:Root	**F = 4.165 , P = 0.029**	F = 0.431 , P = 0.518	F = 0.788 , P = 0.467	CO_2 > Open
Soluble carbohydrates	F = 0.901 , P = 0.419	F = 1.789 , P = 0.194	F = 0.813 , P = 0.455	
Areal soluble carbohydrates	F = 0.765 , P = 0.476	F = 1.749 . P = 0.198	F = 0.816 , P = 0.454	
Sediment OM	F = 1.219 , P = 0.314	F = 0.569 , P = 0.458	F = 1.262 , P = 0.302	
Sediment OC	F = 0.912 , P = 0.416	F = 0.203 , P = 0.656	F = 1.147 , P = 0.335	
Sediment IC	F = 1.776 , P= 0.192	F = 0.420 , P = 0.523	F = 1.742 , P = 0.198	

FIGURE CAPTIONS

Figure 1. Schematic of chamber array. Solid boxes designate an acrylic chamber supplied with either $CO_{2(aq)}$ enriched seawater or $CO_{2(aq)}$ ambient seawater. Dashed boxes designate unmanipulated, open control plots with no chamber. Red and blue line represent $CO_{2(aq)}$ enriched and $CO_{2(aq)}$ ambient seawater, respectively.

Figure 2. Time series of mean pH values at noon within chambered and open control plots throughout the experiment.

Figure 3. Time series of seagrass leaf tissue chemistry %C, %N, and %P within the nutrient unenriched (left column) and nutrient enriched (right column) carbon treatments.

Figure 4. Time series of seagrass leaf C:N, C:P, N:P elemental ratios within the nutrient unenriched (left column) and nutrient enriched (right column) carbon treatments.

Figure 5. Time series of relative leaf growth rates within the nutrient unenriched (left column) and nutrient enriched (right column) carbon treatments.

Figure 6. Seagrass total biomass and shoot:root ratios in July.

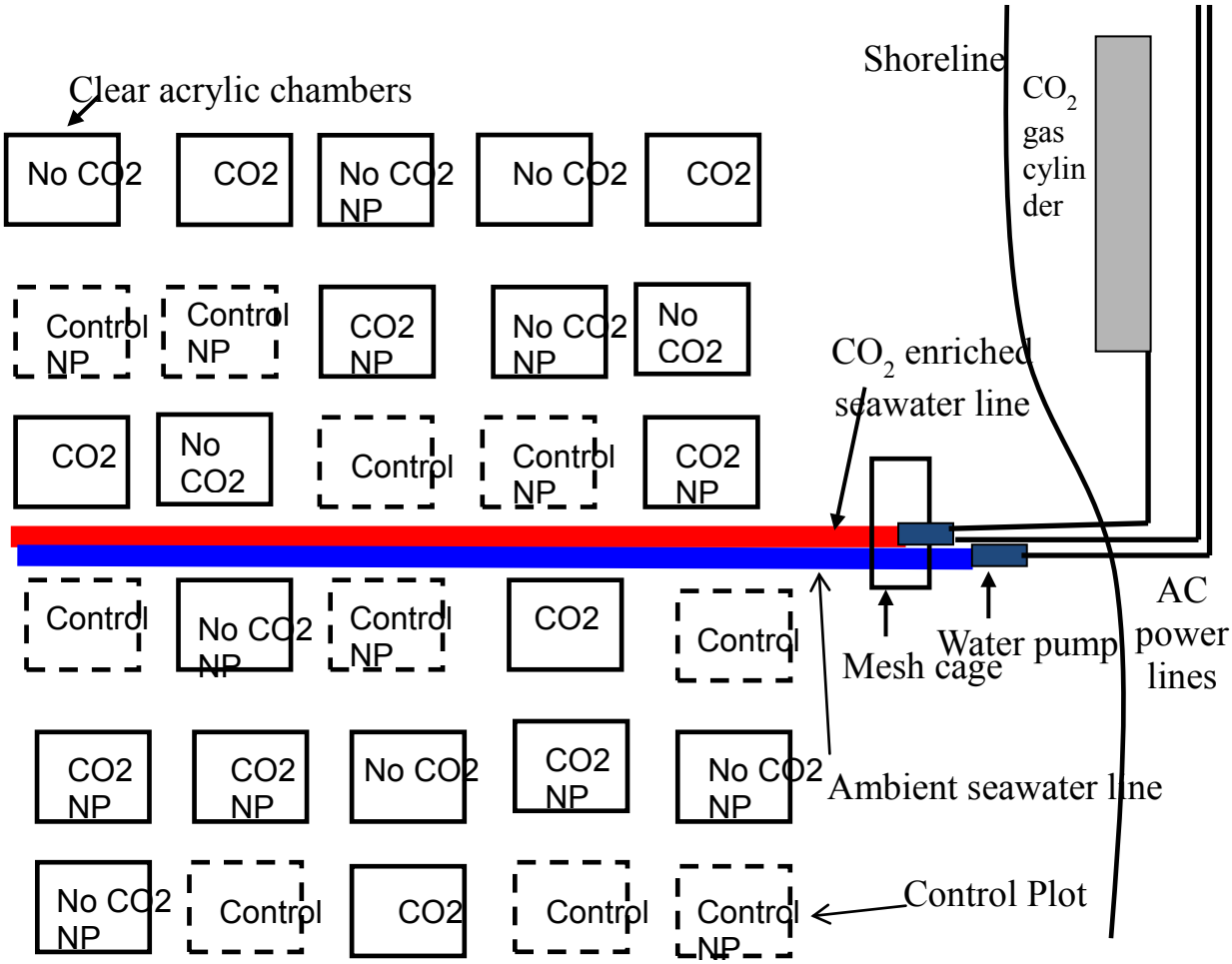
Clear acrylic chambers
Shoreline
CO_2 gas cylinder
CO_2 enriched seawater line
Mesh cage
Water pump
AC power lines
Ambient seawater line
Control Plot
No CO2
CO2
No CO2 NP
Control
Control NP
CO2 NP
No CO2 NP

FIGURE 1.

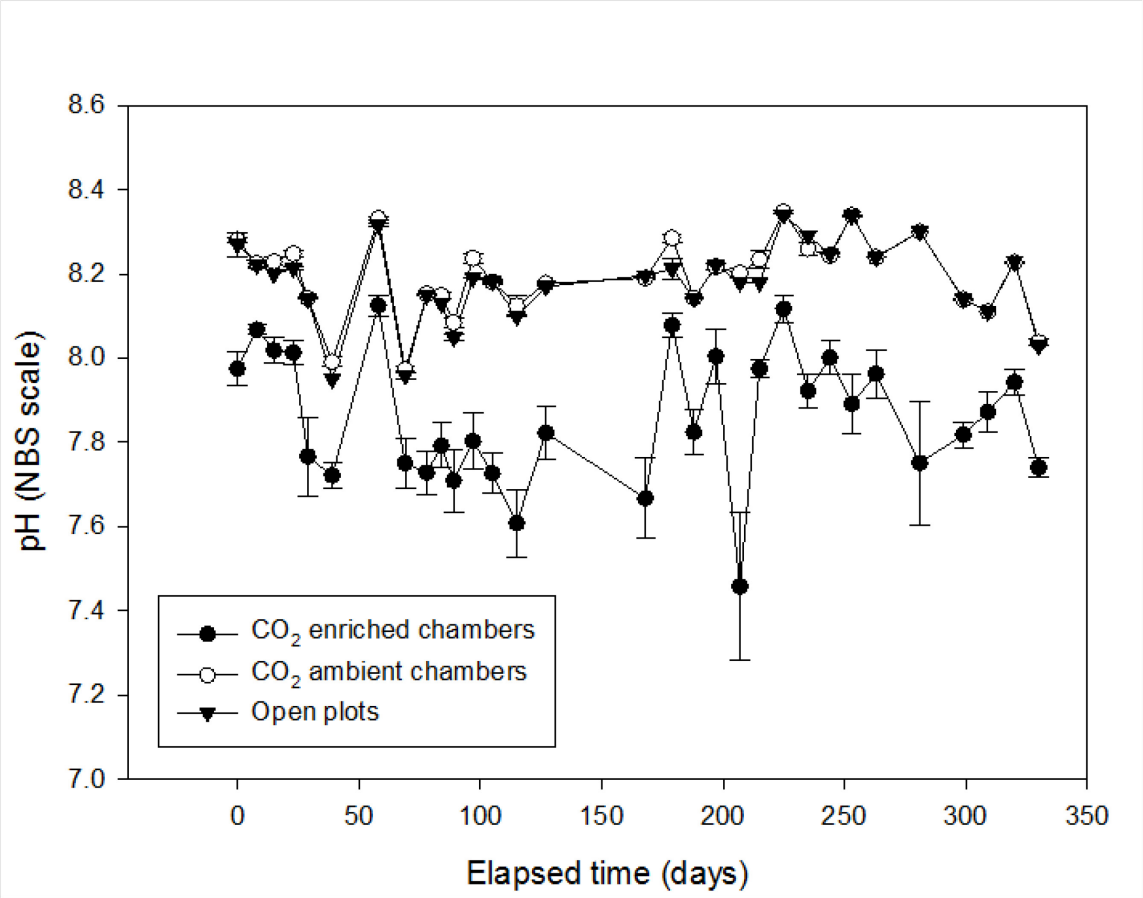
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
pH (NBS scale)
0
50
100
150
200
250
300
350
Elapsed time (days)
CO2 enriched chambers
CO2 ambient chambers
Open plots

FIGURE 2.

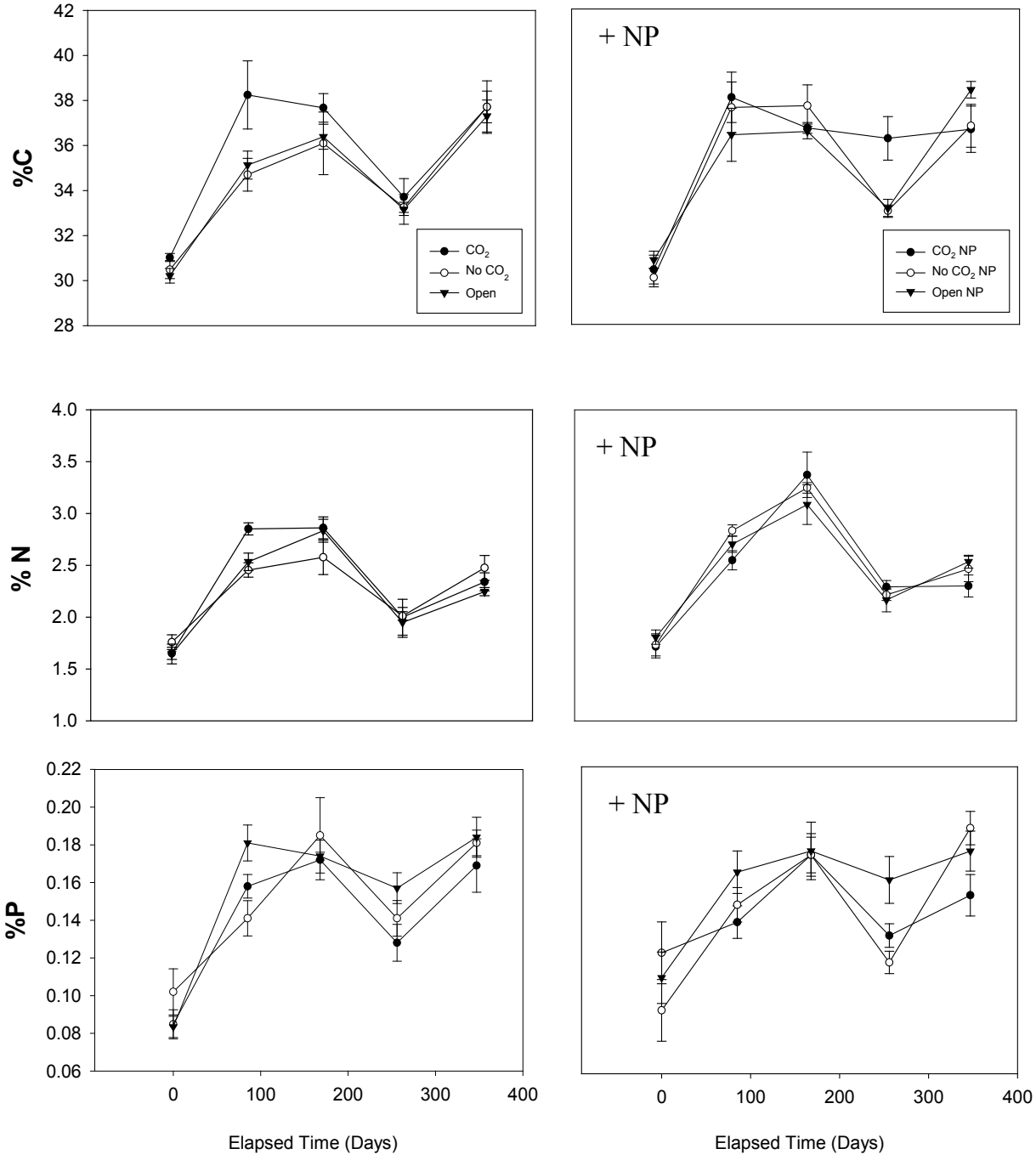
%C
% N
%P
+ NP
+ NP
+ NP
CO2
No CO2
Open
CO2 NP
No CO2 NP
Open NP
Elapsed Time (Days)
Elapsed Time (Days)

FIGURE 3.

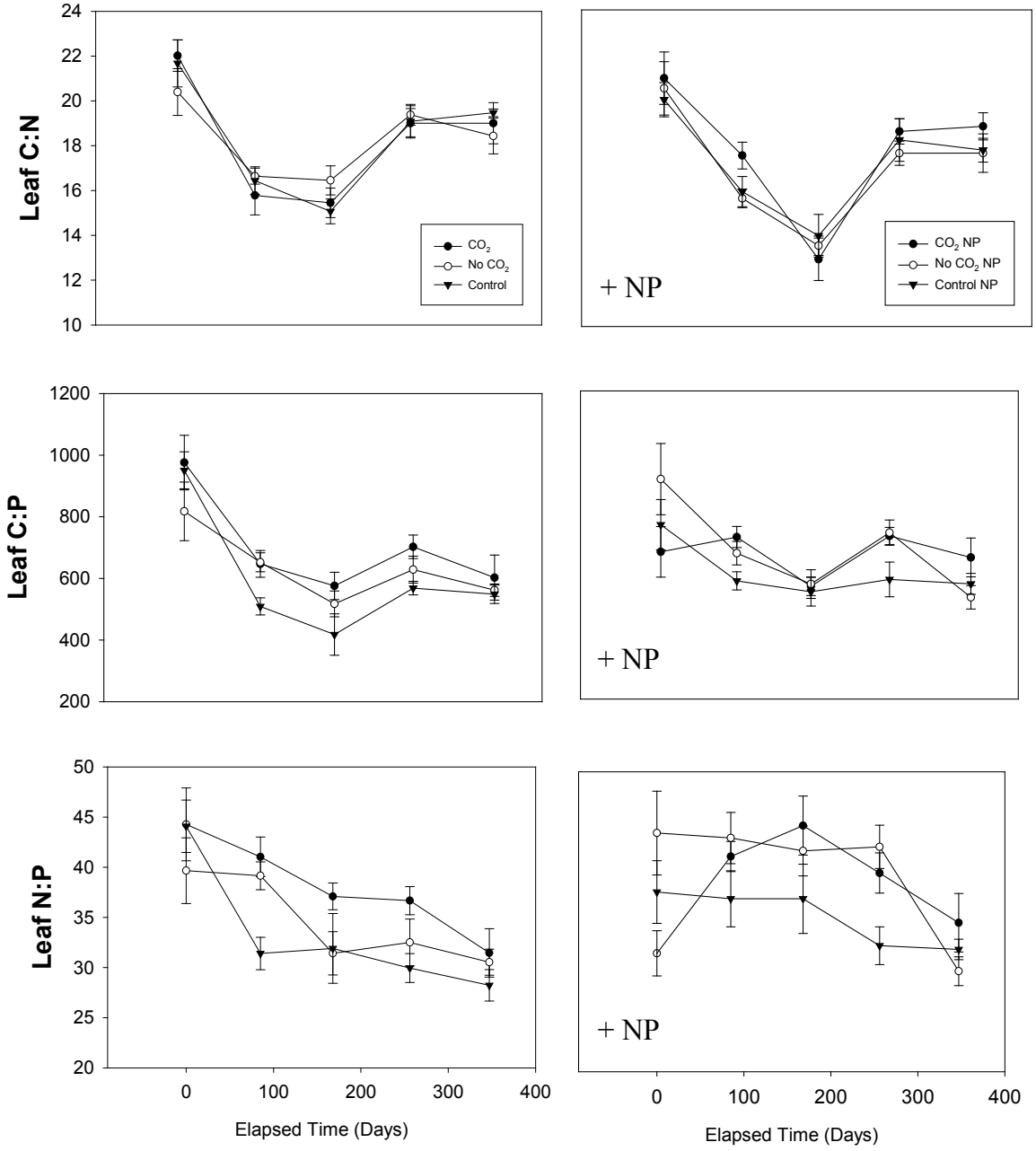
Leaf C:N
Leaf C:P
Leaf N:P
CO2
No CO2
Control
CO2 NP
No CO2 NP
Control NP
+ NP
+ NP
+ NP
Elapsed Time (Days)
Elapsed Time (Days)

FIGURE 4.

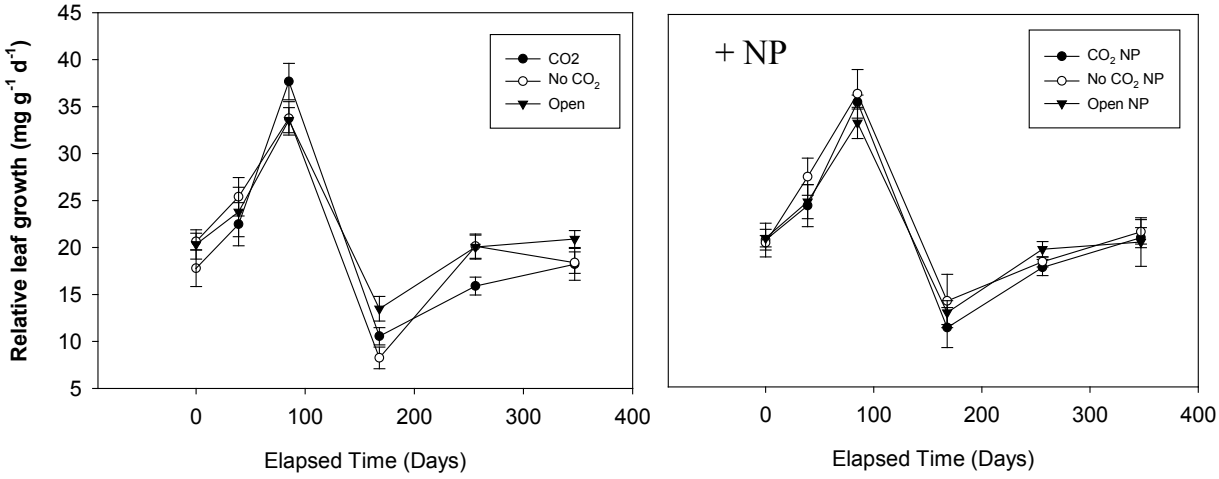
Relative leaf growth (mg g-1 d-1)
45
40
35
30
25
20
15
10
5
0
100
200
300
400
Elapsed Time (Days)
CO2
No CO2
Open
+ NP
CO2 NP
No CO2 NP
Open NP
0
100
200
300
400
Elapsed Time (Days)

FIGURE 5.

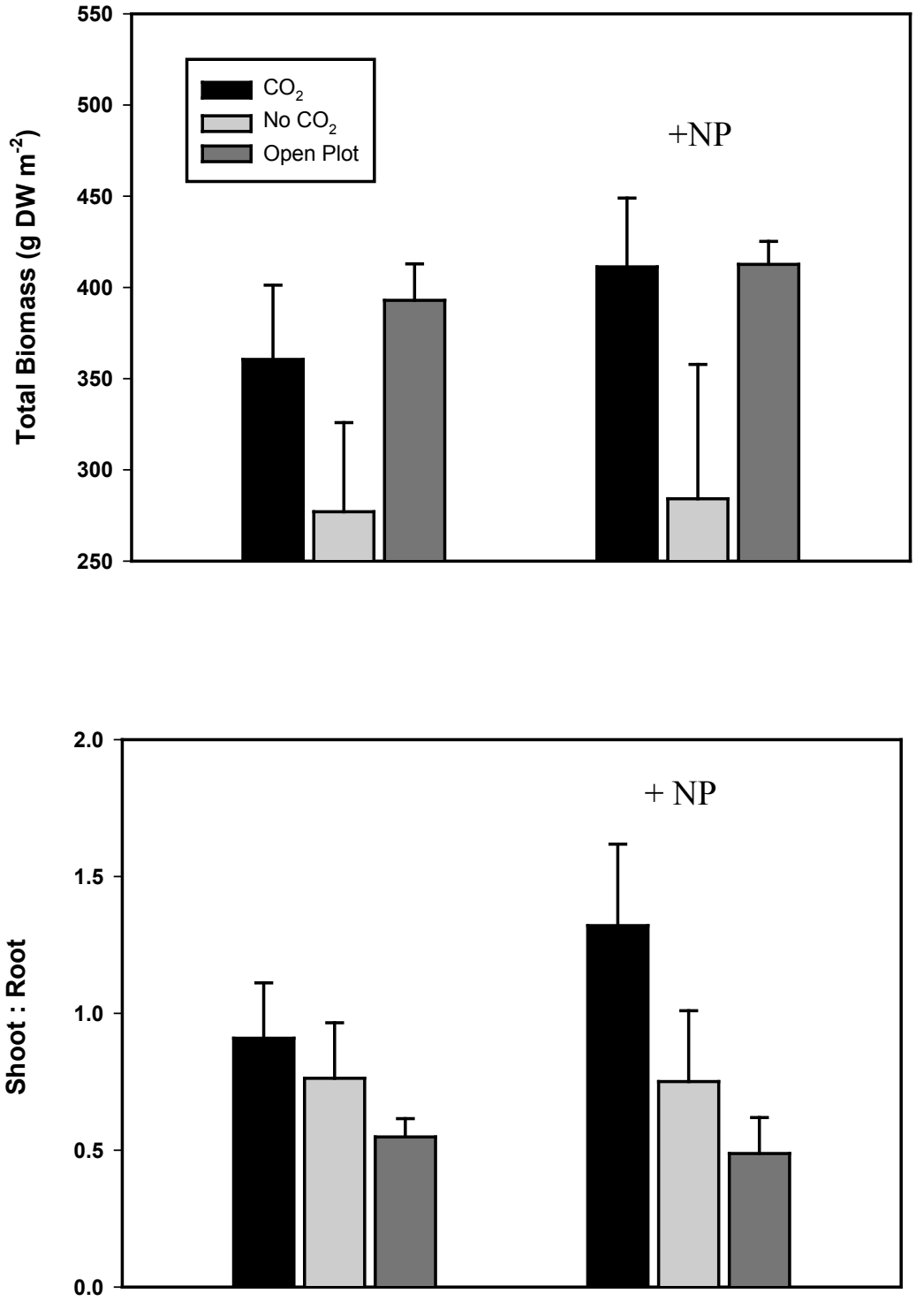
550
500
450
400
350
300
250
Total Biomass (g DW m-2)
CO2
No CO2
Open Plot
+NP
2.0
1.5
1.0
0.5
0.0
Shoot : Root
+ NP

FIGURE 6.

CHAPTER VII

IN SITU CO_2 AND NUTRIENT ENRICHMENT ALTERS THE COMMUNITY STRUCTURE OF SEAGRASS EPIPHYTES

ABSTRACT

Developing a framework for synergistic interactions between multiple anthropogenic stressors remains an important goal in environmental research. In coastal ecosystems, the relative effects of climate change and localized stressors, in combination, have received limited attention. Utilizing novel techniques, we examine the effects of eutrophication and climate-change mediated increases in CO_2 concentrations on the community structure of seagrass epiphytes. A fully factorial experiment was established in a nearshore seagrass bed in the Florida Keys for 11 months. *In situ* CO_2 manipulations were conducted utilizing clear, open-top chambers which regulated seawater carbonate parameters within the seagrass canopy, and replicated dissolved CO_2 forecasts for the year 2100. Nutrient enrichment consisted of monthly additions of granular fertilizer (nitrogen and phosphorus) to the sediments. Seagrass epiphyte community structure was assessed on a seasonal basis, and revealed declines in the abundance of crustose coralline algae (CCA) and concurrent increases in the abundance of filamentous red algae with CO_2 enrichment. These trends resulted in large shifts in the epiphyte community structure from calcified towards fleshy taxa. Epiphyte carbonate load, and the abundance of calcareous polychaete worms followed similar trends, with strong declines under CO_2 enrichment. Carbon dioxide mediated responses in the epiphyte community were most evident during the winter sampling. Nutrient enrichment had no overall effect on seagrass epiphyte load or community structure, and synergistic interactions between CO_2 and nutrient enrichment were not detected. The observed responses in the seagrass epiphyte community suggest that ongoing ocean acidification might strongly influence epiphyte

assemblages in seagrass beds. Such results could have implications towards future rates of biogenic sediment production. Furthermore, seasonal trends in the effects of CO_2 enrichment suggest that future climate change studies need to incorporate larger temporal scales within seasonally variable environments.

INTRODUCTION

Over the next century, it is anticipated that a majority of coastal ecosystems will be affected by a number of anthropogenic stressors. The impact of eutrophication within these widely-distributed marine systems has been a common research theme, with numerous studies based in both basic and applied ecology. While coastal eutrophication has been at the forefront, alternate stressors (such as climate change) are beginning to have an effect, and are gaining importance in terms of how they might alter ecosystem functionality over both short and long time spans. Although eutrophication and climate change represent anthropogenic effects which act in concert, a majority of studies fail to adequately address potential synergistic interactions between these persistent and wide-spread stressors (Harley et al. 2006; Wernberg et al. 2012)

Nutrient addition can strongly alter the structure of plant communities within both terrestrial, aquatic, and marine systems (Smith et al. 1999). Coastal habitats are particularly sensitive to eutrophication because of their close proximity to urban and agricultural development, resulting in increased reports of global declines in the health and integrity of these systems (Orth et al. 2006). Seagrasses, along with associated assemblages of macroalgae and benthic microalgae, comprise an important component of coastal plant communities, which will be altered by eutrophication. Frequently, seagrass

declines associated with increased nutrient inputs occur via shifts in the competitive dominance of various plant taxa. As a result, a common manifestation of eutrophication in seagrass beds is an increase in the abundance of rapidly growing algal taxa which overgrow and shade seagrasses (Duarte 1995). Thus, coastal eutrophication and nutrient enrichment studies aim to provide a framework upon which we can form a predictive understanding of how various plant groups respond to environmental stressors. Prior work has suggested that the abundance of macro- and micro-algal groups might be a function of nutrient availability (Tomasko and Lapointe 1991; Neckles et al. 1993), however other studies have documented weak relationships (Frankovich and Fourqurean 1997; Fourqurean et al. 2010b) and varying site-specific trends (Ferdie and Fourqurean 2004; Armitage et al. 2005). While multiple factors (nutrients and herbivore grazing) have been investigated in regards to the abundance of macro and micro algal groups in seagrass beds, few studies have considered interactions with alternate climate change stressors, which might improve our predictive understanding of how plant groups respond to anthropogenic stressors.

In addition to nutrient loading, increases in dissolved CO_2 concentrations associated with climate change may strongly alter the community composition of seagrass beds. Anthropogenic activities have dramatically increased atmospheric and oceanic CO_2 concentrations, resulting in large changes in seawater carbonate chemistry, particularly within shallow coastal waters. As CO_2 concentrations rise, certain carbonate species increase in abundance ($CO_{2(aq)}$ and HCO_3^-) while other species decrease in abundance (CO_3^{2-}). Associated with these changes, is a reduction in seawater pH, with forecasts of nearly a 0.3 - 0.5 unit reduction by the year 2100 (Caldeira and Wickett

2003). A large majority of macroalgal and seagrass epiphytic taxa produce skeletons with carbonate mineralogy which is sensitive to pH and negatively impacted by seawater acidity (Hall-Spencer et al. 2008; Martin et al. 2008; Porzio et al. 2011). Alternatively, CO_2 enrichment has been shown to increase the dominance of some fleshy algal groups, either by fueling photosynthetic carbon fixation, or releasing them from competition with calcareous groupings (Gao et al. 1999; Kubler et al. 1999). While the single factor of CO_2 enrichment can have drastic consequences for the structure and functionality of algal assemblages, the effects and interactions of concurrent non-climate change stressors needs to be additionally evaluated.

The present study examined the interaction between nutrient and CO_2 enrichment on epiphyte community structure in a tropical seagrass bed, dominated by *Thalassia testudinum*. We assess the relative importance of these 2 stressors in their ability to structure epiphyte community composition. Macroalgal epiphytes are important components of seagrass ecosystems, and substantially contribute to the overall productivity of coastal habitats. Epiphytes are often cited as potential agents in the decline of seagrass systems via overgrowth and shading in response to nutrient enrichment (Tomasko and Lapointe 1991), making them a key component to study in the context of how seagrass systems will respond under simultaneous environmental stressors. We hypothesized that both nutrient and CO_2 enrichment would interactively alter the seagrass epiphyte community. Because epiphyte carbonate can account for as much as 70-80% of the epiphyte standing stock (Frankovich and Zieman 1994), we expected large declines in the abundance of calcareous taxa under CO_2 enrichment. Furthermore, we expected nutrient enrichment to increase seagrass epiphyte loads

overall, and synergistically interact with CO_2 enrichment by further promoting dominance of fleshy epiphyte groups.

METHODS

Study site

In situ manipulation of CO_2 concentrations and nutrient availability was conducted within a shallow (1m depth), nearshore (10m off-shore) seagrass bed in the Florida Keys, Florida, USA (24.55 N, 81.75 W). The benthic community was dominated by the seagrass *Thalassia testudinum*, with sparse abundances of the seagrasses *Syringodium filiforme* and *Halodule wrightii*. Calcareous green algal species (*Halimeda* spp. and *Penicillus* spp.) were additionally present with a patchy distribution. The sediments were composed of roughly 9% organic matter; with the remaining mineral fraction consisting of fine biogenic calcium carbonates.

Experimental design

A balanced, 3 x 2 factorial experiment was designed to study the interactive effects of CO_2 and nutrient enrichment on seagrass epiphytes. The carbon treatment consisted of 3 levels (CO_2 enriched plots, CO_2 ambient plots, and controls), and the nutrient treatment consisted of 2 levels (nitrogen and phosphorus addition, +NP; and control,C). Thirty experimental seagrass plots were arranged in a grid design (5 rows x 6 columns), whereby each column represented a separate complete block. Replicates (n=5) for each treatment were then randomly assigned within each column (Fig. 1). Seagrass plots (.17m^2) were spaced at 1 m intervals throughout the grid.

CO_2 enrichment

Optically-clear, open-top acrylic chambers were used to establish the carbon enriched seagrass plots (see Campbell and Fourqurean (2011) for a detailed description). CO_2 enriched seawater was generated in the field by bubbling pure CO_2 gas into a series of submerged water pumps, which subsequently delivered CO_2 enriched seawater into the carbon enriched chambers via an underwater network of PVC. This technique has been demonstrated as an effective means of confining CO_2 enrichment to a localized area of the benthos. As carbon enriched seawater is pumped into the chambers it cycles within the seagrass canopy before being flushed out the top of the chamber. This design allows for long-term constraint of carbonate parameters within the enriched chambers while limiting the effects of reduced light and water motion. The CO_2 ambient chambers received unenriched seawater from an independent PVC network connected to a separate series of water pumps. Open seagrass control plots lacked chambers, and were designated with a PVC frame. The level of CO_2 enrichment was carefully controlled, and set to mimic forecasts for the year 2100, roughly a 0.3 unit reduction in pH (Caldeira and Wickett 2003). On a weekly basis, all chambers were scrubbed clear of any fouling, and pH measurements (NBS scale, relative accuracy ± 0.002) were taken within all chambers and control plots during the 1200-1500 time period. Water samples were additionally collected periodically during the experiment to monitor salinity and total alkalinity. During each sampling, 20ml of seawater was sampled within each chamber, filtered through a 0.7 um GFF filter, and stored on ice until further processing. Total alkalinity was measured in the lab via automated, potentiometric titration with 0.1 N HCL, and salinity was measured with an Orion conductivity meter. Carbonate parameters ($CO_{2(aq)}$,

HCO_3, CO_3, and calcite/aragonite saturation states) were calculated with the CO_2Sys Excel Macro (Lewis and Wallace 1998), using the dissociation constants of Mehrbach et al (1973), refit by Dickson and Millero (1987).

Nutrient enrichment

Fertilizer was evenly distributed by hand over each designated +NP chamber or plot on a monthly basis. Nitrogen was added in the form of urea-coated slow-release N fertilizer (Polyon, Pursell Technologies; 38-0-0, 94% nitrogen as urea) and phosphorus was added as deflourinated granular phosphate rock [Multifos, IMC Phosphates] $Ca_3(PO_4)^2$, 18%P). Previous studies have effectively used these enrichment methods to increase nutrient availability to both the above- and belowground biomass of benthic macrophytes in South Florida (Ferdie and Fourqurean 2004). Final loading rates were 1.54 g N m^{-2} d^{-1} and 0.24 g P m^{-2} d^{-1}, and were similar to prior eutrophication studies within this region (Armitage et al. 2005; Armitage et al. 2011).

Epiphyte sampling

The seagrass epiphyte community was assessed on a seasonal basis (winter and summer sampling) during the course of the 11 month experiment. During each sampling event, 6 seagrass shoots were randomly harvested from each replicate chamber and control plot, carefully transferred to a zip-lock bag, and placed on ice for transport. Epiphyte abundance was quantified as leaf percent cover using point count software on scanned images. One shoot within each replicate chamber and control plot was randomly selected, leaves were detached from the short shoot with a razor blade, and both sides were scanned at high resolution (1200 dpi) using a Canon flatbed scanner. Image files

were subsequently analyzed by the point count software (CPCe, Kohler, K.E. and S.M Gill 2006), whereby 100 points were randomly superimposed across the leaves of each shoot, and the epiphyte taxa underneath each point was recorded. Analysis revealed that several distinct epiphyte taxa existed at our site, thus epiphyte categories were collapsed into broad groupings as either (crustose coralline algae or filamentous red algae). Epiphyte taxa were recorded as relative proportions of the distributed points for each shoot. Benthic foraminifera and *Spirobis* sp. also comprised part of the epiphyte community and were recorded (individuals/shoot). Epiphyte load was quantified by analyzing the chlorophyll-*a* (Chl *a*) characteristics of the attached epiphytes on 2 randomly selected seagrass shoots from within each plot. Leaves were removed from each short shoot, rinsed in deionized water, and measured for length and width to the nearest mm. The epiphytic material from each shoot was carefully scraped into a single preweighed 20 ml glass scintillation vial. Epiphytes were lyophilized to obtain a dry weight, and then stored in 20 ml of 90% acetone for Chl-*a* extraction for a minimum of 72 hrs. The Chl-*a* content of the extract was measured fluorometrically (Strickland and Parsons, 1972) on a Shimadzu RF- 5301 PC Spectrofluorometer (excitation = 435 nm, emission = 667 nm). Total epiphyte load was quantified as epiphyte dry mass (dry mass/shoot), leaf specific epiphyte mass (epiphyte mass/leaf area), and leaf specific Chl-*a* load (ug Chl-*a* /leaf area). Epiphyte autotrophic index (ug Chl-*a*/ g epiphyte dry mass) was additionally calculated. Epiphyte $CaCO_3$ load was assessed utilizing a gravimetric-acidification technique. The remaining 3 seagrass shoots from each chamber and control plot were rinsed in deionized water, and all leaves were removed from the short shoot with a razor, and dried at 70° C for 48 hrs. Leaves were then weighed, acidified for 3

minutes in 5% HCL, and reweighed. Epiphytic calcium carbonate load (g $CaCO_3$/g dry plant mass) was determined by calculating weight loss after acidification. Subjecting epiphyte-free leaves to similar acidification procedures revealed that weight losses associated with leaching were relatively minor.

Statistical analyses

Seawater carbonate parameters were analyzed by comparing the 95% confidence intervals of the mean carbonate measurements (recorded during the 1200-1500 time period throughout the experiment) within the chambers and control plots. Seasonal measurements of epiphyte abundance, epiphyte Chl-*a* characteristics, and epiphyte carbonate load were analyzed with a repeated measures, two-way ANOVA; with season as the within subject factor, and CO_2 and nutrient enrichment as the between subject factors. When significance was detected, post hoc analysis was performed with a Holm-Sidak test, whereby the overall significance level was adjusted to 0.05 to account for multiple comparisons. During each sampling event, all replicate, subsampled measurements within each chamber and control plot were averaged to avoid pseudoreplication. All data were tested for normality and variance homogeneity.

RESULTS

Seawater parameters

Temperature and salinity averaged 29.1 °C and 35.5 respectively, during the course of the experiment. Total alkalinity was recorded during 7 distinct dates throughout the experiment and averaged 2503 umol kg^{-1}. Calculations of the seawater carbonate parameters revealed that CO_2 enrichment was effectively maintained within the enriched

chambers for the duration of the experiment, and remained within parameter forecasts for the year 2100 (Table 1). Mean pH measurements within the CO_2 enriched chambers (for both levels of nutrient addition) were 7.88, while mean pH within the unenriched chambers and control plots was 8.20 and 8.19 respectively. Seawater carbonate calculations further revealed that CO_2 concentrations were 2.5 times higher, and CO_3^{2-} concentrations were 1.7 times lower within the enriched chambers as compared to the controls. Calcite and aragonite saturation states were strongly altered by CO_2 enrichment, were additionally 1.7 times lower within the enriched chambers as compared to the controls.

Epiphyte abundance and $CaCO_3$ load

The epiphyte community was dominated by a collection of coralline red algae (*Melobesia membranacea* and *Hydrolithon farinosum*) and filamentous red algae (*Ceramium brevizonatum* and *Polysiphonia binneyi*.). The polychaete worm *Spirobis* sp. and an unidentified foraminifera were also present. Thus, percent cover data contained 4 epiphytic categories (crustose coralline algae, filamentous red algae, polychaete worms, and foraminifera). Other unidentified epiphytes represented less than 1% of the taxa at our site on a percent cover basis.

The abundance of crustose coralline algae (CCA hereafter) was altered by CO_2 enrichment ($p < 0.001$) and season ($p = 0.029$) (Fig. 2, Table 2). The interaction between these 2 factors was also significant ($p < 0.001$), demonstrating that CO_2 enrichment reduced CCA abundance only during the winter. Overall CCA abundance was higher in the winter than in other seasons, however only within the control chambers and open

plots. Nutrient enrichment had no impact on CCA abundance in either season ($p = 0.1$). Epiphytic $CaCO_3$ load followed similar trends to CCA abundance (Fig. 3).

Filamentous red algae abundance increased in response to CO_2 enrichment ($p < 0.01$), and was unaffected by nutrient enrichment ($p = 0.7$)(see Fig. 4). Post-hoc analysis revealed that the CO_2 enriched chambers were distinct from both the ambient chambers ($p=0.057$) and the control plots ($p=0.01$). Season had no effect on filamentous red algae abundance, and treatment interactions were not significant.

The abundance of the polychaete worm (*Spirobis* sp.) was reduced with CO_2 enrichment ($p < 0.01$, Fig 5, Table 2), yet unaffected by nutrient enrichment. *Spirobis* abundance declined during the summer sampling and furthermore, CO_2 enrichment had a significantly greater effect during the winter season; thus the within subjects factor of season, and the interaction between season and CO_2 were significant ($p < 0.05$ and $p < 0.01$, respectively).

Foraminifera abundance was reduced by the presence of the chambers, thus the between subjects factor of carbon was significant ($p<0.01$), however there were no significant differences between the CO_2 enriched and unenriched chambers (Fig. 6, Table 2). The within subjects factor of season was significant ($p < 0.01$), as was the interaction between season and carbon treatment ($p < 0.001$), suggesting that seasonal differences were greater within the open plots, as compared to the chambered treatments.

Epiphyte Chl-a characteristics

Epiphyte Chl-*a* loads (ug Chl-*a* / cm leaf area) were significantly higher during the summer season compared to winter ($p < 0.001$, Fig. 7), however neither CO_2,

nor nutrient enrichment had any effect on epiphytic loading. Dry epiphyte mass (g epi mass/shoot) and leaf specific epiphyte mass (g epi mass/cm leaf area) were not significantly impacted by season, nutrient or CO_2 enrichment (Table 2). Epiphyte autotrophic index (ug Chl-*a*/ g epiphyte dry mass) was additionally not impacted by CO_2 or nutrient enrichment, however season did have a significant impact ($p < 0.01$), with higher values during the summer sampling (Table 2).

DISCUSSION

In situ CO_2 and nutrient enrichment of a nearshore seagrass bed revealed that epiphyte community structure can be strongly regulated by CO_2 addition alone. Experimental CO_2 enrichment reduced the abundance of calcareous taxa (CCA and *Spirobis*), while increasing the abundance of fleshy filamentous reds. Thus, CO_2 mediated shifts in the community structure of seagrass epiphytes might be anticipated with ongoing ocean acidification over the next century. Furthermore, seasonal dynamics revealed that the effects of CO_2 enrichment can vary depending upon the sampling period, thus future ocean acidification studies should make efforts to repeatedly sample across time. Contrary to our predictions, nutrient enrichment had no effect on epiphyte load or community structure, suggesting that alternate factors regulate the seagrass epiphyte community at this location.

The calcareous skeleton of crustose coralline algae renders them as a potentially sensitive taxa towards acidified conditions, as demonstrated in prior laboratory and field experimentation (Hall-Spencer et al. 2008; Jokiel et al. 2008; Kuffner et al. 2008; Martin et al. 2008; Gao and Zheng 2010). Our findings support these conclusions, as we

document a decline in CCA percent coverage from 20% in the control treatments to only about 1% under acidified treatments during the winter sampling. Epiphytic calcium carbonate loads followed similar trends, with substantial declines under acidified conditions. Crustose coralline algae produce carbonate skeletons with a highly soluble mineralogy (high-magnesium calcite), which more readily dissolves under acidic conditions as compared to other forms of $CaCO_3$; thus, future ocean acidification might have drastic consequences for these taxa (Jokiel et al. 2008). In shallow, seagrass dominated systems, crustose coralline algae can substantially contribute to the mud sized fraction of biogenic carbonate sediments (Frankovich and Zieman 1994), thus our data suggest that ocean acidification might strongly alter carbonate sediment production within these systems.

Seasonal variation in both CCA abundance and the effects of CO_2 enrichment within our experiment might be attributed to multiple factors; however, the limited sampling frequency (twice per annum) within this experiment limits our ability to draw strong conclusions from this trend. While overall CCA abundance and epiphyte $CaCO_3$ load were lower during the summer sampling, we detected no seasonal change in epiphyte dry mass (g / shoot), nor any changes in leaf specific epiphyte mass (g / cm leaf area). Contrary to the declines in CCA abundance, we documented increases in Chl-*a* load and epiphyte autrophic index during the summer. The summer increase in epiphyte autotrophic index suggests a decrease in the contribution of calcified taxa towards total Chl-*a* loads, and is likely driven by summer reductions in CCA abundance. Overall, such trends might suggest temporal shifts in the dominance of one epiphyte group relative to another at our site, with CCA taxa dominating in the winter, and alternate, non-calcified

groups contributing more during the summer. The overall decline in the representation of calcified groups in the summer may account for the significant season x CO_2 interaction, and a reduced effect of CO_2 enrichment during the summer season.

The abundance of the epiphytic polychaete worm, *Spirobis* sp. was significantly reduced with CO_2 enrichment, and unaffected by nutrient addition. The mechanisms for these CO_2 induced declines in abundance are likely similar to those responsible for declines in CCA abundance. *Spirobis* worms secrete protective $CaCO_3$ shells which are sensitive to acidification, similarly demonstrated by declines in the abundance of calcareous polychaetes along natural pH gradients (Cigliano et al. 2010). Benthic foraminifera abundance was not influenced by CO_2 enrichment, however this epiphyte group was significantly altered by the presence of our chamber design. These unexpected findings may have been due to the slight reduction (<5%) in light levels imposed by our chambers, as many benthic foraminifera host photosynthetic algal symbionts (Hallock and Peebles 1993). Thus, conclusions in regards to the insensitivity of benthic foraminifera to CO_2 enrichment within this experiment should be interpreted with caution. We note however that the chamber design had no effect on other photosynthetic epiphyte taxa within our experiment.

Carbon dioxide significantly influenced the abundance of filamentous red epiphytes. The CO_2 enriched chambers displayed higher abundances as compared to both the ambient chambers and open control plots, and such trends were not dependent upon season or nutrient load. Carbon dioxide induced increases in non-calcified algal groups (particularly turf taxa) have been demonstrated in several studies (Russell et al. 2009; Connell and Russell 2010), thus our findings generally support these conclusions.

Overall, non-calcified algal taxa have been generally considered unresponsive to CO_2 enrichment because of the use of efficient carbon concentrating mechanisms (CCMs), and the demonstration of carbon saturated photosynthetic rates at current CO_2 levels (Beer and Koch 1996). However, prior work has established that not all algal groups contain and utilize CCMs (Maberly 1990; Maberly et al. 1992; Raven 2003), thus it is anticipated that these groupings may in fact benefit from increases in external CO_2 supply (Hepburn et al. 2011). Mesocosm manipulations of CO_2 strongly increased the biomass and abundance of fleshy, non-calcified temperate algal turfs (Russell et al. 2009; Connell and Russell 2010). These growth responses were linked to increases in photosynthetic effective quantum yield (Fv/Fm), suggesting CO_2 enrichment improved photosynthetic performance within these algal taxa. A similar mechanism may have operated in our experiment, and account for our CO_2 related increases in fleshy epiphytes. Furthermore, prior work has suggested that most algal taxa which lack CCMs (predominantly relying upon the diffusive flux of CO_2) were members of the Rhodophyta (Maberly et al. 1992; Raven et al. 2002a). Within this context, the filamentous red algae at our experimental site might have benefitted from additional CO_2 because of the lack of efficient CCMs. Further experimentation will be required, and we suggest that future ocean acidification research might benefit by directing efforts towards studying how members of Rhodophyta respond to increases in CO_2 concentrations.

Our study additionally supports prior work aimed at addressing shifts in algal community structure under climate change stressors (Hall-Spencer et al. 2008). In addition to being potentially stimulated by CO_2 supply, the dominance of filamentous red epiphytes may have resulted from a decreased competition for space with CCAs, as

suggested by previous research (Russell et al. 2009; Hepburn et al. 2011). While our sampling methodology prevents us from drawing strong conclusions about the role of space competition in our observed shifts in community composition, we submit that CO_2 addition may have further favored filamentous red algae by decreasing CCA abundance, and increasing the space available for recruitment on the surface of the seagrass leaf blades.

Seagrass epiphyte Chl-*a* loads were not altered by either CO_2 or nutrient enrichment. We also failed to detect any treatment effects on dry epiphyte mass (per shoot) or leaf specific epiphyte mass. Thus, while we document large shifts epiphyte community structure, epiphyte loads remained similar among our treatments. Epiphyte loading has been commonly cited as cause of seagrass decline due to excessive overgrowth and shading by the epiphyte community (Duarte 1995). The similarity of loading metrics (Chl-*a*, dry epiphyte mass, and leaf specific epiphyte mass) among our treatments suggests that any improvement in light transmission to the leaf surface due to CO_2 induced declines in CCA were offset by increases in filamentous red algae.

Nutrient enrichment had no effect on any of our measured epiphyte metrics, suggesting that alternate factors likely control the seagrass epiphyte community at our site. Our findings support the conclusions of previous studies which suggest that epiphyte responses to nutrient enrichment can be variable, and site specific within this region (Armitage et al. 2011). Additional work has further documented weak relationships between nutrient availability and epiphyte loads across Florida Bay and the Florida Keys (Frankovich and Fourqurean 1997; Fourqurean et al. 2010b). Epiphyte communities can be strongly structured by top-down forces (Neckles et al. 1993; Heck and Valentine

2006), thus any increase in epiphyte loading as a result of nutrient enrichment may have been mediated by consumers at our experimental site (Gil et al. 2006).

Synergistic interactions between CO_2 enrichment and nutrient supply were largely undetected in our experiment. These findings contrast with other studies aimed at addressing interactions between climate change and other stressors. Russell et al. (2009) documented interactions between CO_2 and nutrient enrichment on the recruitment of temperate turf algae to unoccupied space. Additional CO_2 increased turf recruitment only with nutrient enrichment, and this interaction provided a 34% increase in recruitment as compared to the sum of the individual effects. The lack of significant carbon x nutrient interactions, and the disparities between Russell et al. (2009) and this study might result from methodological distinctions between *ex situ* and *in situ* experimentation. Our experimental design allowed grazers full access to our experimental plots, thus nutrient (and carbon x nutrient interactions) may have been mediated by the presence of natural grazers, highlighting the importance of conducting climate change research under realistic, natural environments. We suggest that future climate change research will largely benefit when *in situ* experimentation is paired to laboratory and mesocosm studies.

Our work documented the effects of CO_2 enrichment relative to nutrient enrichment on the community structure of seagrass epiphytes. By addressing stressor interactions in coastal seagrass ecosystems we can begin to evaluate the impact of a full suite of pressures which are anticipated to grow in the near future. Our work demonstrates that CO_2 enrichment alone can shift the community structure of seagrass epiphytes, supporting the paradigm of declines in sensitive calcareous taxa, coupled to

increases in fleshy, non-calcified taxa. Furthermore, these effects were most prominent during the winter season, suggesting that future climate change research will need to address broader temporal scales, and reduce restrictions to limited time frames. The lack of a nutrient response from the seagrass epiphyte community suggests that the effects of eutrophication might be spatially variable, potentially depending upon interactions with available consumers (an important variable to consider for future enrichment studies) (Heck and Valentine 2006). Our experimental design highlights the increased need for *in situ* experimentation, providing a realistic interpretation of the effects and interactions of multiple climate change stressors on ecological systems.

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Table 1. Seawater carbonate parameters observed within the chambered and open control plots. Values represent the averages of repeated measurements (1200-1500 time frame) taken during the course of the 11 month enrichment experiment. Bracketed values represent 95% confidence intervals. During the experiment total alkalinity averaged 2503 umol kg^{-1}, while temperature and salinity averaged 29.1° and 35.5 respectively.

Seawater parameter	Control NP	Control	No CO_2 NP	No CO_2	CO_2 NP	CO_2
pH (NBS scale)	8.19 (8.22-8.16)	8.19 (8.22-8.15)	8.19 (8.22-8.16)	8.20 (8.23-8.17)	7.90 (7.95-7.84)	7.86 (7.92-7.81)
DIC (umol/kg SW)	2140 (2190 - 2089)	2140 (2191-2090)	2136 (2185-2087)	2132 (2180-2083)	2305 (2360-2250)	2319 (2374-2263)
CO_2 (umol/kg SW)	11.5 (12.7-10.3)	11.6 (12.8-10.4)	11.3 (12.4-10.2)	11.1 (12.2-10.0)	27.4 (32.6-22.3)	29.9 (35.0-24.8)
HCO_3 (umol/kg SW)	1865 (1922-1808)	1866 (1924-1809)	1860 (1914-1805)	1853 (1907-1798)	2119 (2181-2057)	2138 (2200-2075)
CO_3 (umol/kg SW)	263 (280-246)	262 (280-245)	265 (281-249)	268 (284-252)	159 (176-141)	151 (169.6 - 132.6)
PCO_2 (uatm)	445.4 (488.7-402.0)	447 (490-404)	437 (476-398)	429 (468-390)	1054 (1243-864)	1147 (1329-965)
Ω Calcite	6.4 (6.8-6.0)	6.3 (6.8-5.9)	6.4 (6.8-6.0)	6.5 (6.9-6.1)	3.8 (4.3 - 3.4)	3.7 (4.1-3.2)
Ω Aragonite	4.3 (4.5-4.0)	4.2 (4.5-4.0)	4.3 (4.6-4.0)	4.3 (4.6-4.1)	2.6 (2.9-2.3)	2.4 (2.8-2.1)

Table 2. Summary of statistical results (2-way, repeated measures ANOVA, a= 0.05) of measured epiphyte community metrics. Bold values indicate significant effects ($p < 0.05$). Post hoc comparisons for the carbon factor were conducted with a Holm Sidak test (overall significance at $p < 0.05$). Similar letter superscripts indicate no significant difference between respective CO_2 levels (CO_2 enriched chamber; CO_2 ambient chamber; open control plots).

Source of Variation	CCA abundance	Filamentous red abundance	Spirobis abundance	Foraminifera abundance	Epiphyte $CaCO_3$ load	Chl a load	Dry epiphyte mass	Leaf specific epiphyte mass	Autotrophic index
within subjects factor									
season	**0.021**	0.254	**0.044**	**< 0.001**	**< 0.001**	**0.019**	0.733	0.357	**< 0.001**
season x carbon	**0.01**	0.658	**0.007**	**0.001**	**0.002**	0.228	0.69	0.454	0.296
season x nutrient	0.95	0.983	0.505	0.515	0.624	0.233	0.428	0.357	0.158
season x carbon x nutrient	0.993	0.834	0.663	0.889	0.396	0.567	0.635	0.725	0.707
between subjects factor									
carbon	**< 0.001** ^{a,b,b}	**< 0.001** ^{a,b,b}	**0.005** ^{a,b,ab}	**< 0.001** ^{a,a,b}	**0.001** ^{a,b,b}	0.426	0.218	0.419	0.351
nutrients	0.277	0.979	0.2	0.41	0.926	0.201	0.261	0.19	0.814
carbon x nutrients	0.925	0.897	0.082	0.225	0.701	0.7	0.398	0.71	0.55

FIGURE CAPTIONS

Figure 1: Schematic of experimental design. Solid boxes indicate clear acrylic chamber designated as either carbon enriched (CO_2) or unenriched (No CO_2). Dashed boxes indicate open control plots which lack chambers. Chambers and plots and nutrient additions are additionally designated.

Figure 2: Visual percent coverage (means ± 1SE) of crustose coralline algae (CCA) growing on leaves of *T. testudinum*.

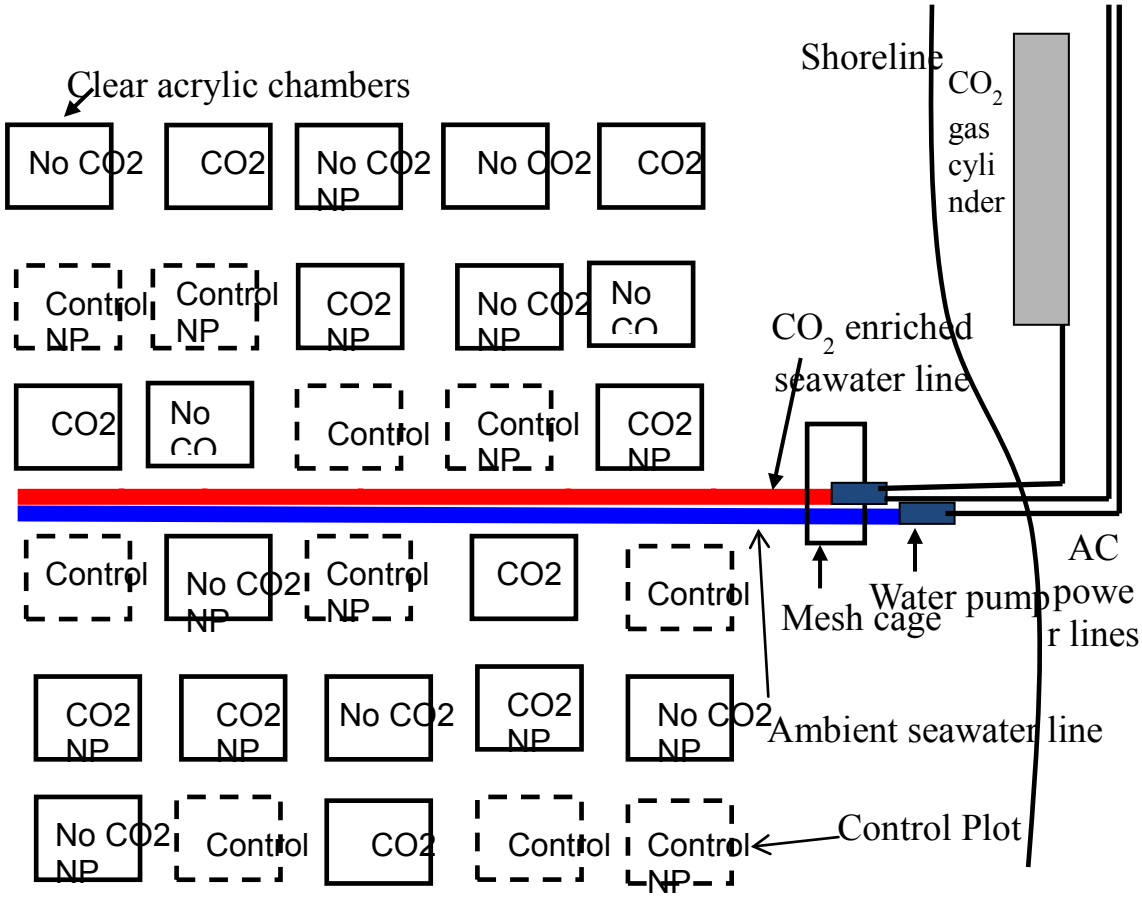
Figure 3: Epiphytic $CaCO_3$ load (means ± 1SE) on the leaves of *T. testudinum*.

Figure 4: Visual percent coverage (means ± 1SE) of filamentous red algae growing on leaves of *T. testudinum*.

Figure 5: Abundance (means ± 1SE) of polychaete worms (*Spirobis* sp.) growing on the leaves of *T. testudinum*.

Figure 6: Abundance (means ± 1SE) of benthic foraminifera growing on the leaves of *T. testudinum*.

Figure 7: Epiphyte chlorophyll-a load (means ± 1SE) of *T. testudinum* leaves.


Clear acrylic chambers
Shoreline
CO_2 gas cylinder
CO_2 enriched seawater line
Mesh cage
Water pump
AC power lines
Ambient seawater line
Control Plot
No CO2
CO2
No CO2 NP
Control
Control NP
CO2 NP
No CO2 NP
No CO
Control
Control NP
CO2 NP
CO2
No CO
Control
Control NP
CO2 NP
Control
No CO2 NP
Control NP
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No CO2
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No CO2 NP
No CO2 NP
Control
CO2
Control
Control NP

FIGURE 1.

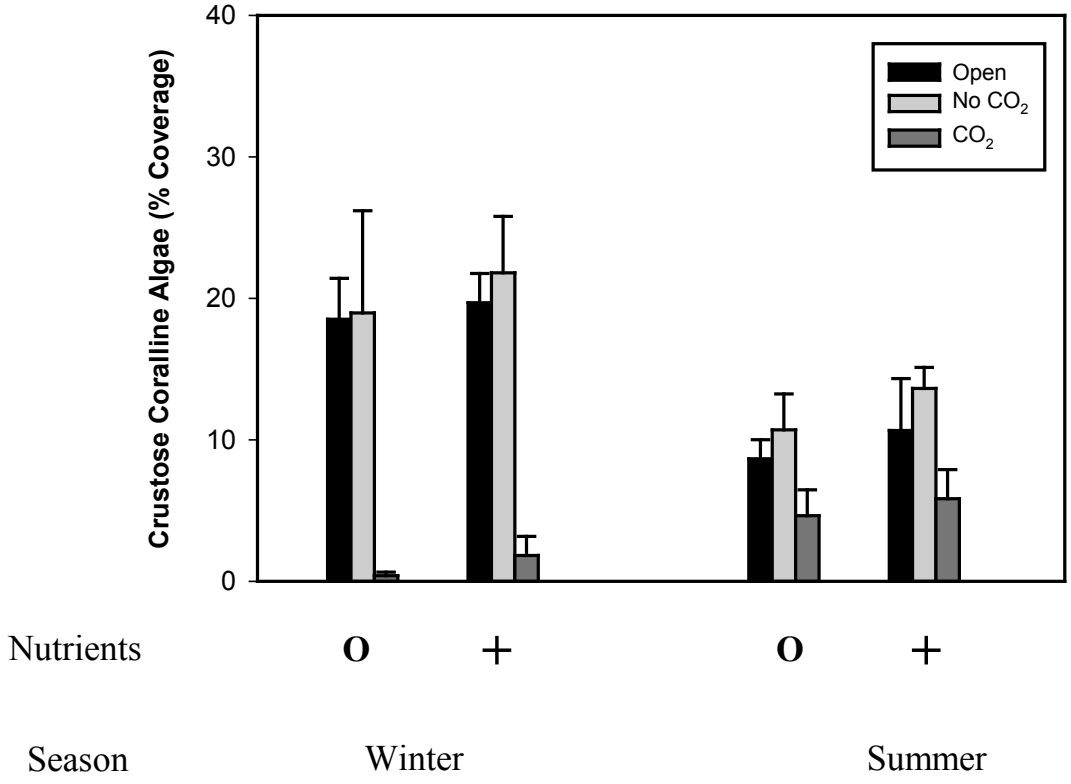
Crustose Coralline Algae (% Coverage)
40
30
20
10
0
Open
No CO2
CO2
Nutrients
O
+
O
+
Season
Winter
Summer

FIGURE 2.

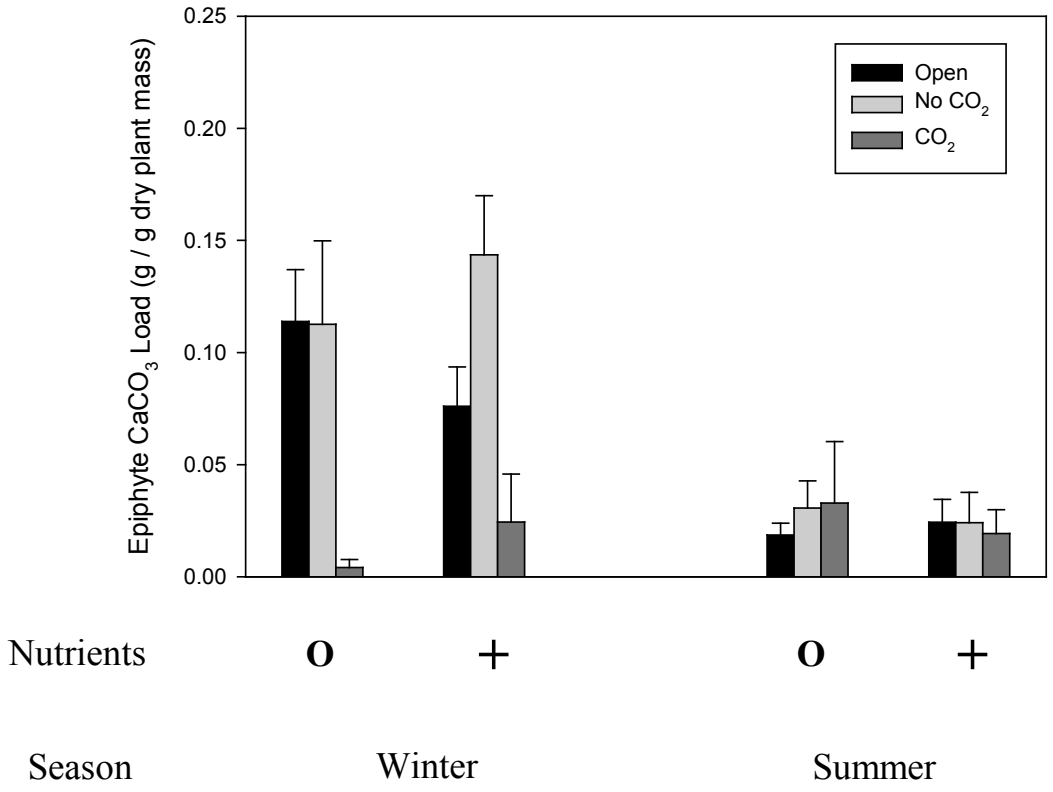
0.25
0.20
0.15
0.10
0.05
0.00
Epiphyte $CaCO_3$ Load (g / g dry plant mass)
Open
No CO_2
CO_2
Nutrients
O
+
O
+
Season
Winter
Summer

FIGURE 3.

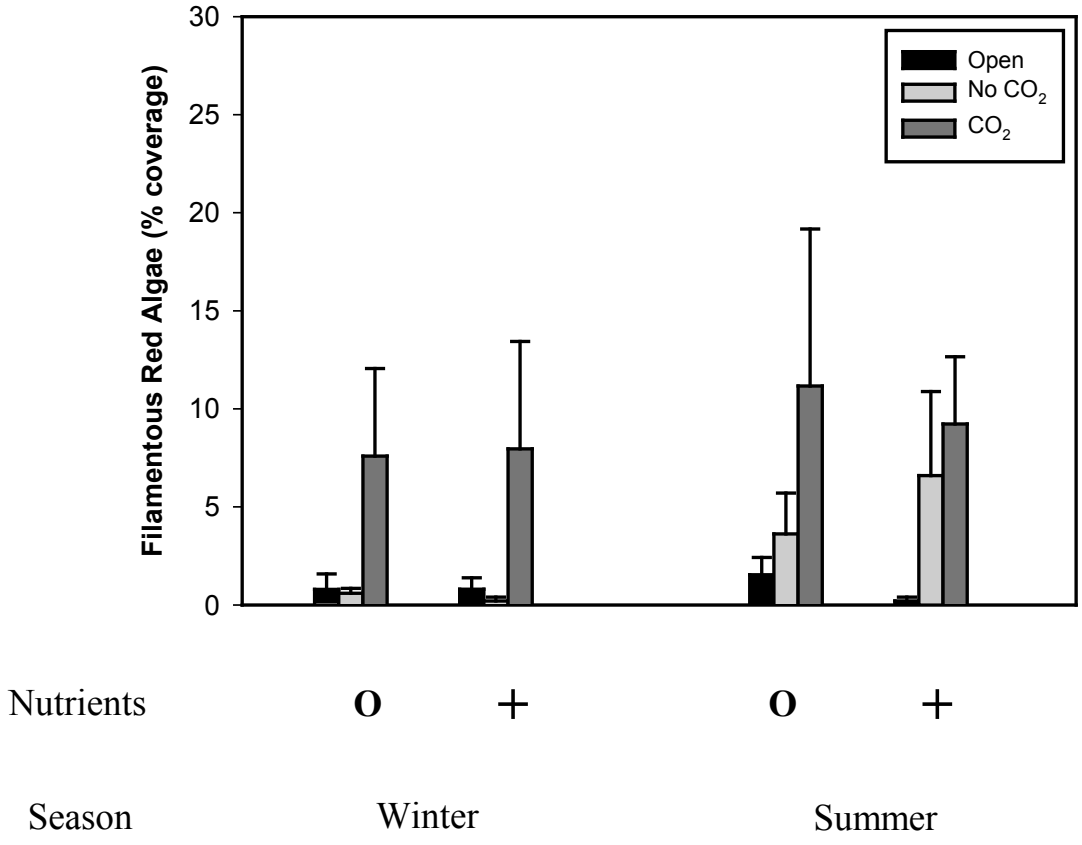
Filamentous Red Algae (% coverage)
30
25
20
15
10
5
0
Open
No CO_2
CO_2
Nutrients
O
+
O
+
Season
Winter
Summer

FIGURE 4.

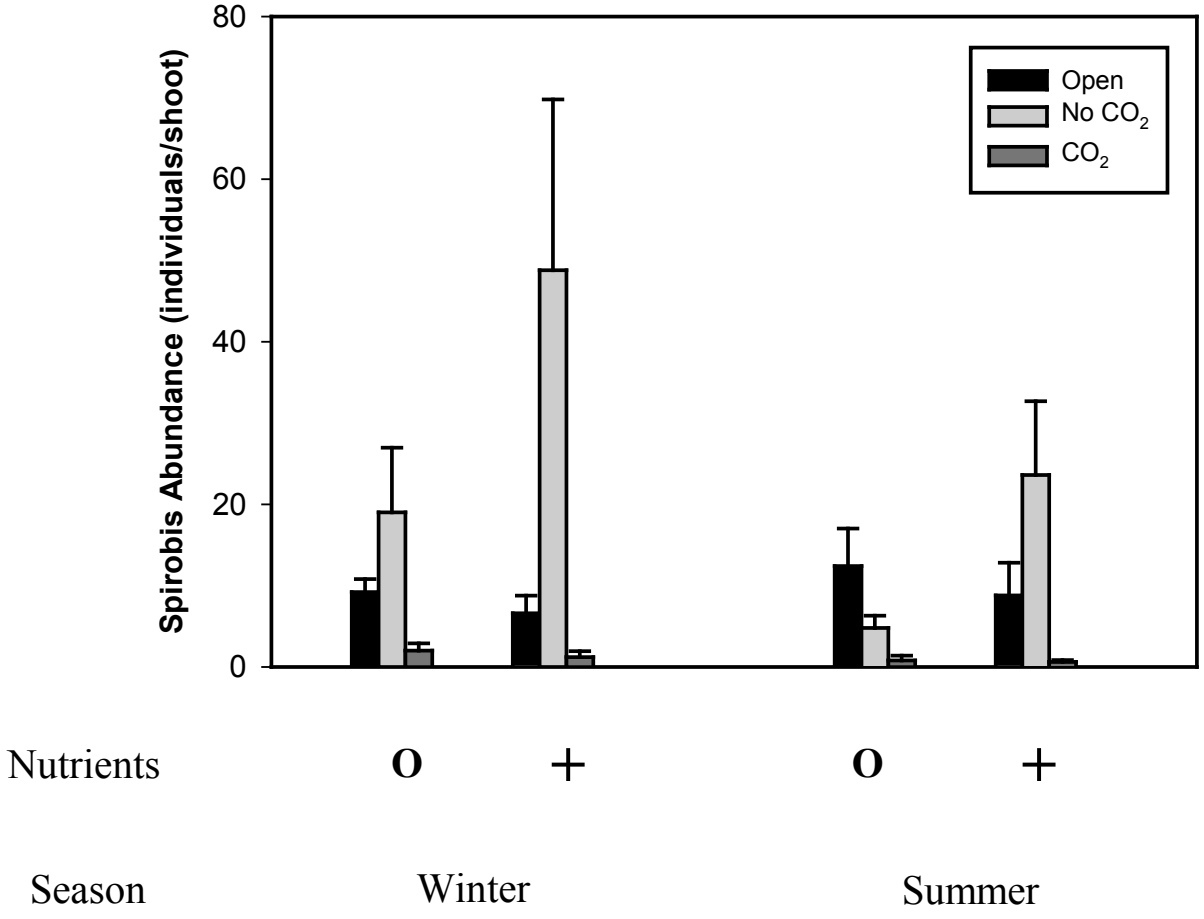
Spirobis Abundance (individuals/shoot)
80
60
40
20
0
Open
No CO_2
CO_2
Nutrients
O
+
O
+
Season
Winter
Summer

FIGURE 5.

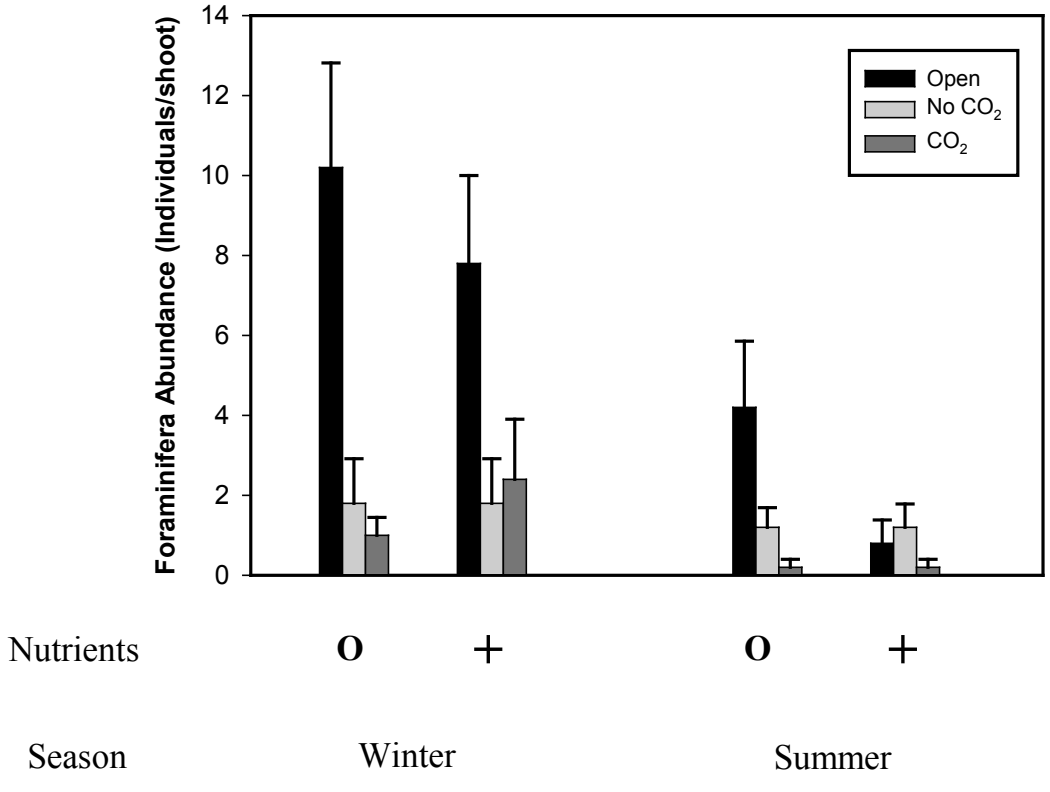
Foraminifera Abundance (Individuals/shoot)
14
12
10
8
6
4
2
0
Open
No CO_2
CO_2
Nutrients
O
+
O
+
Season
Winter
Summer

FIGURE 6.

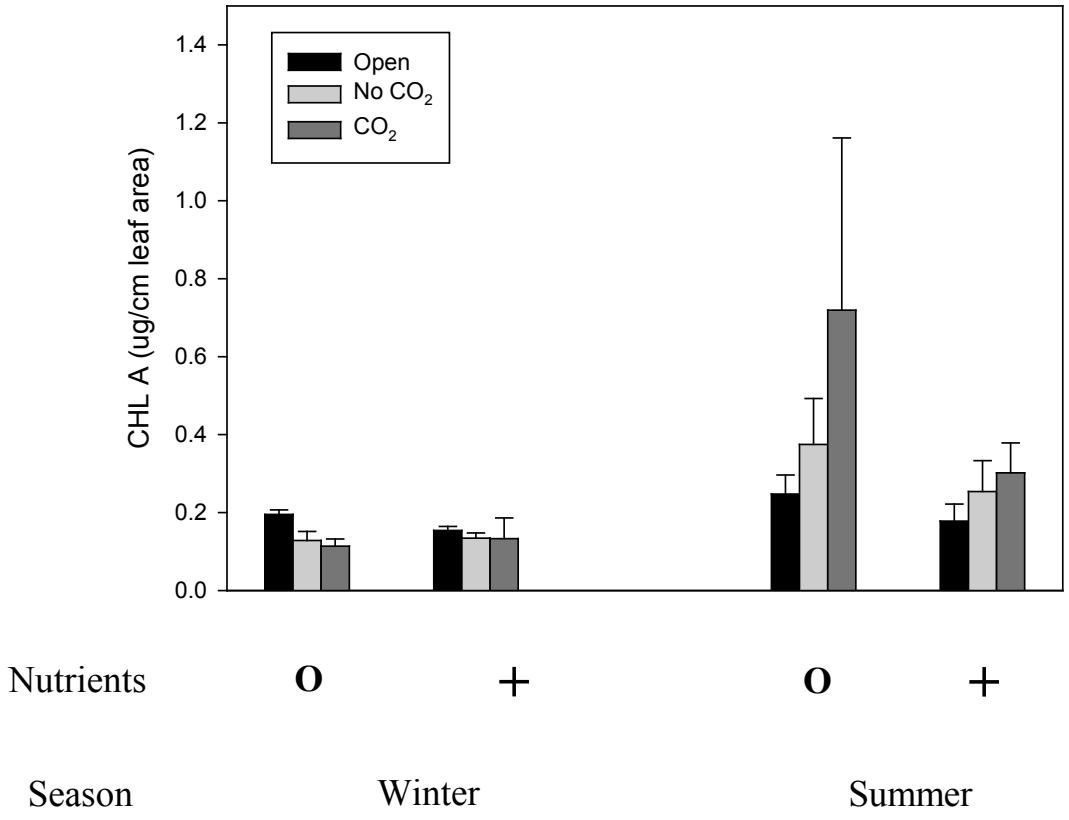
Open
No CO2
CO2
CHL A (ug/cm leaf area)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Nutrients
O
+
O
+
Season
Winter
Summer

FIGURE 7.

CONCLUSIONS AND FUTURE DIRECTIONS

Natural ecosystems will likely be strongly impacted by rising CO_2 levels. Currently, photosynthetic rates are commonly undersaturated with respect to present day CO_2 concentrations, and as a result, a wide variety of plants display increases in photosynthetic efficiency with elevated CO_2 (Drake et al. 1997). Terrestrial research has demonstrated that these small scale physiological responses can have larger implications towards plant community structure, net primary production, carbon storage, and nutrient cycling (Bazzaz 1990; Luo et al. 2006).

Seagrasses represent a group of marine plants which will also be exposed to increasing CO_2 concentrations. Laboratory research has demonstrated that photosynthetic rates for these submerged plants are similarly undersaturated with respect to present day concentrations of dissolved CO_2, and they display rapid increases with elevated CO_2. While terrestrial research has advanced due to studies which link CO_2 responses across multiple spatial scales, seagrass CO_2 research currently lacks such research attempting to address larger scale responses. The similarities between the photosynthetic responses of terrestrial and marine plants suggest that marine systems may additionally display a number of large scale responses to elevated CO_2 concentrations. The goals of this dissertation were to develop and implement techniques aimed at expanding the scope and scale of seagrass CO_2 enrichment research. Our study addressed the long term *in situ* responses of an intact seagrass bed to CO_2 enrichment, and provides an assessment of whether small scale responses documented in laboratory studies are realized under natural environmental conditions. Here, I present a summary of major conclusions, and suggest directions for future work.

It is clear that functionality of seagrass systems will likely change as a result of increasing CO_2 concentrations. Chapters I and II document short-term photosynthetic responses, while chapters IV and VI link these small-scale responses to larger-scale changes in plant nutrient content, carbohydrate content, and overall seagrass biomass. In general, we find valid support for prior *ex situ* seagrass CO_2 research, and suggest a number of mechanisms worth exploring for future experimentation.

Findings from chapters IV and VI suggest a number of transient 'time dependent' responses of CO_2 enrichment on the nutrient and carbohydrate content of seagrasses. During the course of a 6 month enrichment period, elevated CO_2 concentrations decreased plant nutrient content and increased carbohydrate content, similar to the findings of prior terrestrial research. Future seagrass CO_2 research may benefit by further examining the implications of these chemical changes as it relates to rates of grazing and resilience within these systems. Changes in leaf tissue chemistry, either via increased phenolic production or reduced nutrient content, can directly influence seagrass grazers; thus future work might examine how herbivory responds to CO_2-mediated shifts in seagrass chemical composition, following research directives of terrestrial CO_2 research (Bezemer and Jones 1998). Our documented increases in soluble carbohydrates might have strong implications for the ability of these plants to endure disturbance events such as prolonged shading. The benefits of CO_2 enrichment on seagrass resilience clearly require additional study.

Conclusions from chapter VI reveal that over extended time scales, CO_2-mediated increases in seagrass biomass might be realized, supporting conclusions from prior work (Palacios and Zimmerman 2007; Hall-Spencer et al. 2008). Because seagrasses are now

being recognized as global hotspots for long term carbon storage (Fourqurean et al. 2012), future research should be directed towards studying the ultimate fate of this increased production, and whether seagrass ecosystems will increase rates of carbon sequestration and burial under elevated CO_2.

This work did not document any influence of nutrient availability on seagrass response to additional CO_2, contrary to prior hypotheses based upon shifts in seagrass nutrient content (chapter IV) and negative relationships between seagrass nutrient and carbohydrate content (chapter V). These findings suggest that, similar to terrestrial research, the responses of plant systems to increased CO_2 supply may be increasingly complex at larger spatial and temporal scales (Bazzaz 1990). Future work will benefit from studies which continue to examine seagrass responses to long-term CO_2 enrichment over a wide range of environmental gradients. While the role of nutrient availability will certainly require additional research, alternate environmental factors also need to be considered. Seawater temperatures are anticipated to increase along with CO_2 concentrations. Because of the influence of temperature on a number of plant metabolic processes (Zimmerman et al. 1989), examining CO_2 responses across thermal gradients may prove critical towards improving our understanding of how these systems might respond to climate change in the long term.

Lastly, seagrass ecosystems are comprised of an assemblage of primary producers, thus seagrass CO_2 research which examines the responses of entire communities will lead to a more comprehensive understanding of how additional CO_2 will impact these systems. Chapter VII provides insight towards the responses of seagrass epiphytes to *in situ* CO_2 enrichment, and demonstrates rapid responses to carbon

enrichment. Similar to prior research, we document large declines in the abundance of calcified groups, and concurrent increases in fleshy groups. These shifts suggest that future rates of calcification in seagrass systems might strongly decline with increased CO_2 concentrations, potentially altering rates of sediment production. As it seems likely that calcified algae will strongly respond to elevated CO_2, future studies need to consider the implications of reduced calcification rates.

In conclusion, this dissertation documents the effects of CO_2 enrichment on the functionality of seagrass ecosystems across multiple spatial and temporal scales. Responses were observed in a variety of primary producers for several components of the seagrass community. These submerged plants are widely distributed, and provide a range of ecosystem functions; thus documenting the effects of climate change aids in our ability to further evaluate the future role of seagrasses in coastal areas around the globe.

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PUBLICATIONS

Campbell, J.E., L. Yarbro, J. Fourqurean. 2012. Relationships between the nutrient and carbohydrate content of the seagrass *Thalassia testudinum*. *Aquatic Botany* 99:56-60.

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INVITED SEMINARS

2011 - Acidic Seas: The Impacts of Ocean Acidification on Coastal Environments. Biscayne Bay National Park, Miami, FL.

2011 - Seagrass Responses to *In Situ* Carbon Dioxide Enrichment: An Ecological Perspective. Smithsonian Marine Station, Fort Pierce, FL.

PRESENTATIONS

Campbell, J.E. and James W. Fourqurean. 2012. Functional changes in tropical seagrasses at ecologically relevant increases in CO_2 availability. CO_2/OA effects on seagrass meadows: COST workshop. University of Nottingham, UK.

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