Photochemical efficiency of *Thalassia testudinum* varies in response to repeated shading events and unpredictable weather

Melissa A. Genazzio, Michael J. Durako*

Department of Biology and Marine Biology, Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin Moss Ln., Wilmington, NC 28409, USA

ABSTRACT: A diurnal regression approach was recently proposed as an alternative to point measurements of effective photochemical efficiency of photosystem II ($\Delta F/F_m'$) for assessing seagrass physiological condition at the landscape scale. We conducted a mesocosm study, which assessed the responsiveness of the diurnal regression approach and timescales of photoacclimation of *Thalassia testudinum* to repeated week-long cycles of reduced irradiance (Shade treatment), followed by full sunlight. Shade-treatment shoots exhibited photoacclimation responses 1 to 3 d after shades were added or removed in maximum photochemical efficiencies (F_v/F_m), estimates of F_v/F_m derived from $\Delta F/F_m'$ vs. photosynthetically active radiation (PAR) diurnal regression intercepts, and diurnal regression slopes. Estimated F_v/F_m values were more variable but generally only significantly different from measured F_v/F_m during stressful light or hyposalinity conditions, indicating a higher sensitivity of photosystem II to environmental stress under light pressure. Compared to unshaded controls (Sun treatment), the Shade-treatment plants exhibited 26% shorter leaves, a 10% reduction in leaf width, a 43% decrease in leaf area and a 50% decrease in dry weight after 47 d. Shade treatments also showed an increase in leaf chlorophyll *a* and *b* content with a decrease in chlorophyll *a:b* ratio. These patterns suggest that Shade-treatment plants were light-limited. Significant day-to-day variation in measured and regression-derived estimates of photochemical efficiency in response to overcast and hyposaline conditions reflected rapid changes in chlorophyll fluorescence in response to short-term weather changes. This day-to-day variation frequently equaled or exceeded treatment responses, suggesting that the use of diurnal regressions of $\Delta F/F_m'$ vs. PAR may not be an effective method for assessing seagrass physiological health at landscape scales.

KEY WORDS: *Thalassia testudinum* · Photochemical efficiency · Light reduction · Photoacclimation · Seagrass monitoring

INTRODUCTION

Thalassia testudinum Banks ex König is the dominant seagrass species in Florida Bay, USA (Zieman 1982). A rapid and widespread die-off of seagrasses in 1987 to 1990 resulted in the loss of almost 30% of the *Thalassia*-dominated communities in Florida Bay, which was followed by a cascade of negative ecological effects (Robblee et al. 1991, Butler et al. 1995, Durako et al. 2002, Madden et al. 2009). Owing to

concerns about the status of the seagrass communities in Florida Bay following the die-off, the South Florida Fish Habitat Assessment Program (FHAP-SF) was initiated in 1995. FHAP-SF is a status and trends program that monitors the distribution and abundance of benthic macrophytes (seagrasses and macroalgae) across Florida Bay (Durako et al. 2002). In 2001, FHAP-SF began to monitor the photosynthetic health of *T. testudinum* annually (during May to June) using pulse-amplitude modulated (PAM)

fluorometry, *in situ*, in 10 shallow basins across Florida Bay (Durako & Kunzelman 2002, Belshe at al. 2007, 2008).

The main parameter PAM fluorometry measures at ambient irradiances is effective photochemical efficiency ($\Delta F/F_m'$) of photosystem II (PSII). $\Delta F/F_m'$ is defined as $F_m' - F/F_m'$, where F is the minimum chlorophyll fluorescence determined using non-actinic red light flashes and F_m' is the maximum chlorophyll fluorescence measurement after the supersaturating light pulse. Diurnal variations in $\Delta F/F_m'$ and relative electron transport rates (rETR) depend on light history prior to the saturating pulse (Schreiber et al. 1986, Ralph et al. 1998, Beer & Björk 2000, Durako & Kunzelman 2002, Belshe et al. 2007, 2008, Durako 2012, Schubert et al. 2015). Maximum photochemical efficiency of PSII (F_v/F_m, where $F_v = F_m - F_0$; F_m and F_0 correspond with F_m' and F, but under dark acclimation) measures the potential photochemistry of PSII of a plant under dark-acclimated conditions. F_v/F_m yields higher fluorescence than $\Delta F/F_m'$ as non-photochemical quenching (NPQ) is usually absent, although it may take several hours for NPQ to relax fully (Enríquez et al. 2002). Although F_v/F_m is considered a better indicator of stress and recovery than $\Delta F/F_m'$, it is not always feasible to measure F_v/F_m in landscape-scale ecosystem monitoring because of the required dark-acclimated times (typically 10 to 30 min, but see above) (Beer et al. 1998, 2001, Durako & Kunzelman 2002, Enríquez et al. 2002, Ralph & Gademann 2005, Belshe at al. 2007).

When measuring seagrass chlorophyll *a* fluorescence at the landscape scale, seagrasses are exposed to a wide range of irradiances throughout the day and among days (Durako & Kunzelman 2002, Belshe et al. 2007, Cayabyab & Enríquez 2007). Other natural variations such as depth, weather conditions, tides and canopy structure can make it difficult to determine photosynthetic health accurately from point PAM fluorometer measurements over a diurnal period (Falkowski et al. 1990, Durako & Kunzelman 2002, Enríquez et al. 2002, Belshe et al. 2007). Durako (2012) suggested that the use of diurnal regressions of $\Delta F/F_m'$ vs. PAR (photosynthetically active radiation), rather than individual measurements, may provide a more accurate assessment of photochemical efficiency averaged over large spatial and temporal scales. More negative regression slopes indicate the plants are less efficient at dissipating excess energy, reflecting low light acclimation or stress due to increased NPQ. Durako (2012) also suggested that *y*-intercepts of the regression (i.e. when PAR is equal to 0) may provide estimates of

maximum photochemical efficiency (F_v/F_m). Subsequently, Howarth & Durako (2013a) provided experimental support, using *T. testudinum* seedlings, that intercepts from regressions of $\Delta F/F_m'$ vs. PAR may serve as proxies for F_v/F_m under non-stressful conditions. Intercepts and measured F_v/F_m exhibited similar variation patterns in response to salinity and light treatments, but were sometimes significantly different under the most stressful treatment conditions (low light and hypo- or hypersalinity).

Photochemical efficiencies do not always directly correspond to growth or biomass production rates in seagrasses (Beer & Björk 2000, Silva et al. 2009). Thus, comparing PAM data with other parameters such as leaf growth rate, leaf biomass and chlorophyll content should allow a more accurate assessment of plant responses to environmental variation. However, conducting growth measurements simultaneously with PAM fluorometry *in situ* is destructive, time-consuming and may not always be logistically possible.

Here, we report on the results of a mesocosm study using mature short shoots of *T. testudinum*, which were exposed to repeated 1 wk pulses of reduced light followed by 1 wk recovery periods. This study had 3 research objectives. First, we wanted to determine if the diurnal regression parameters suggested by Durako (2012) would detect significant responses to pulsed light stress. Second, we sought to determine the time scales for photoacclimation in *T. testudinum* to pulsed light reductions. Finally, we wanted to determine if changes in photochemical efficiencies or regression parameters co-varied with more classical leaf growth measurements in response to pulsed light stress. The overall goal was to determine if day-to-day variations in weather conditions (i.e. sunny vs. cloudy or rainy days) significantly affect directly measured or diurnal regression-derived estimates of F_v/F_m and to assess time scales of leaf photoacclimatory responses. Our results suggest that both estimates of photochemical efficiency were highly sensitive to short-term variations in light (and salinity). Thus, the efficacy of PAM fluorometry may be limited for assessing seagrass physiological health in typical large-scale field monitoring programs.

MATERIALS AND METHODS

Sample collection

Thalassia testudinum short shoots were collected from dense seagrass beds (Braun-Blanquet values of

3 to 4, indicating 25 to 75% bottom cover; Durako et al. 2002) in Blackwater Sound, FL (25.1692°N, 80.4231°W), at the end of the FHAP-SF sampling on May 31, 2013. Whole mature and apparently healthy shoots (no necrotic lesions on the rank 1 and 2 leaves) of similar leaf blade lengths (≈10 to 15 cm mean length) and leaf numbers (≈3) were collected with roots and at least 5 cm of rhizome attached. Sediments were washed and removed from shoots before preparing for transportation. To avoid possible pseudoreplication, individual shoots were collected at least 2 m apart. Because *T. testudinum* is a clonal plant, seedlings are sometimes used in experimental studies to provide independent replicates (Kahn & Durako 2006). However, the seed provides an additional carbon reserve, which may buffer shoot responses under low light conditions (Howarth & Durako 2013a). The short shoots were transported the next day in coolers with ambient water to the University of North Carolina's (UNCW) Center for Marine Science (CMS) Wilmington, NC, USA.

At CMS, the collected shoots were cleaned of epiphytes and debris, planted in opaque plastic tubs (Rubbermaid; 30 × 38 × 23 cm) filled with aragonite shell hash, placed in polyethylene mesocosms (1.2 × 1.2 × 0.6 m) with continuous seawater and allowed to acclimate for 4 d to account for handling stress (pretreatment). Each rhizome was trimmed to ~5 cm length before the short shoots with roots were planted in the aragonite shell hash. We have maintained *T. testudinum*, initiated from seedlings, in aragonite shell hash in this flow-through seawater system in our CMS greenhouse for over 12 yr.

Experimental design

The experimental design consisted of 4 replicates of 2 light treatments (Sun vs. Shade) for a total of 8 mesocosms. Four randomly assigned mesocosms received full sun (Sun treatment), and 4 received ~50% reduced surface irradiance (Shade treatment) when neutral density screens were added after the acclimation period. Each mesocosm contained 3 tubs, each containing 4 randomly assigned short shoots. The 4 shoots inside each tub represented subsamples within one experimental unit (n = 4 per treatment, level of replication is the mesocosm). To minimize disturbance of adjacent shoots during various sampling, one tub provided shoots for photosynthetic measurements, one tub provided shoots for growth measurements and the third tub provided shoots for pigment extraction. After the 4 d pre-treatment

period, one shoot from both the pigment and the morphometrics tubs in each mesocosm was sacrificed and analyzed to provide initial leaf chlorophyll content and leaf area, respectively. After pre-treatment, the first of 3 treatment cycles was initiated. Each treatment cycle consisted of a week-long shading period where irradiance was reduced after screens were placed over the top of the 4 Shade-treatment mesocosms, followed by a week-long recovery period where the shades were removed.

Each mesocosm had flow-through seawater pumped from the Intracoastal Waterway, Wilmington, NC (5 exchanges d^{-1}). Salinity was measured 6 times daily concurrent with fluorescence measurements (06:00, 09:00, 12:00, 15:00, 18:00 and 21:00 h) using a conductivity meter (YSI model 80). In addition, daily average salinity was calculated from UNCW's CMS conductivity logger (YSI model 6820) measured at the seawater pumping station every 30 min. Water temperatures of each mesocosm were monitored at 30 min intervals with Hobo temperature loggers (Onset). Surface irradiance of each mesocosm and irradiance at the level of the leaf canopy of both Sun and Shade treatments were measured at 15 min intervals with scalar quantum PAR sensors (LiCor LI-193S) attached to a LiCor LI-1400 datalogger. Battery failure in the data-logger from experimental Days 10 to 16 resulted in lost canopy PAR data. Interpolation for missing PAR was made using UNCW's CMS logged surface irradiance data with a cosine-corrected quantum PAR sensor (Apogee Instruments SQ-110). LiCor surface irradiance data were correlated with CMS logged data for the duration of the experiment. A regression coefficient of CMS vs. Licor PAR data was determined for each treatment. Total daily PAR was calculated using trapezoidal interpolation from the logged LiCor PAR every 15 min from 05:00 to 21:00 h.

Chlorophyll fluorescence

To minimize within-shoot variation (Durako & Kunzelman 2002, Enríquez et al. 2002), leaf photochemical efficiency of PSII (F_v/F_m or $\Delta F/F_m'$) was measured at the middle (sheath to leaf tip) of the rank 2 leaf of each of the 4 shoots in the photosynthesis tubs using a PAM fluorometer (Diving-PAM, Walz). A universal sample holder (Diving-USH) was used to maintain a constant distance (5 mm) between the PAM optical fiber and the blade. Measurements were taken at least every other day at 06:00, 09:00, 12:00, 15:00 and 18:00 h to assess diurnal variation of $\Delta F/F_m'$ of PSII

under ambient light. Fluorescence measurements at 21:00 h provided dark-acclimated maximum photochemical efficiencies of PSII (F_v/F_m) for each shoot (Howarth & Durako 2013a).

Chlorophyll extraction

After the final recovery period, shoots in the pigment tubs were harvested for chlorophyll levels and compared to the pre-treatment measurements to determine treatment effects on leaf chlorophyll content, following methods in Durako et al. (2003). Rank 2 blades were collected, cleaned of any epiphytes, and the middle 1 cm section was ground in a chilled mortar and pestle under low light in 90% acetone. Absorbance spectra (280 to 750 nm) of the acetone extracts were measured using an Ocean Optics USB4000 fiber optic spectrometer. Chlorophyll a and b contents were calculated using the following equations, modified from Jeffrey & Humphrey (1975):

$$\text{Chl } a \text{ (µg g}^{-1}\text{ FW)} = \{[11.93 \times (A_{664} - A_{750}) \\ - 1.93 \times (A_{647} - A_{750})] \times \text{acetone vol}\}/\text{FW}$$

$$\text{Chl } b \text{ (µg g}^{-1}\text{ FW)} = \{[21.03 \times (A_{647} - A_{750}) \\ - 5.43 \times (A_{664} - A_{750})] \times \text{acetone vol}]\}/\text{FW}$$

where FW is the fresh weight (g) of the leaf tissue; A is absorbance of light; and acetone vol is the amount (ml) extracted.

Leaf characteristics

After the final recovery period, shoot-specific leaf number and leaf area were measured from shoots in the morphometric tub for both treatments and compared to the pre-treatment measurements to determine treatment effects on leaf growth. Leaf necrosis is an indicator of leaf deterioration (Romero et al. 2007) and was quantified during morphometric measurements into 2 categories: 'green', showing less than 25% necrotic tissue (usually present as small brown spots or streaks along the leaf margin), and 'brown', showing at least 75% necrotic tissue. Length and width for each tissue type (green or brown) were measured using a metric ruler, and one-sided leaf area (cm²) was calculated as the sum of the area (length × width cm) of each blade per shoot. Following measurement, leaves were dried at 60°C for 48 h to determine dry weight.

Leaf growth (g dry wt [DW] per leaf per day) was assessed using the leaf-punch technique (Zieman 1974) during 5, week-long intervals, during the ex-

periment (Cycle 1 shading period, Cycle 2 shading and recovery periods, and Cycle 3 shading and recovery periods). Leaf length, width and dry weights were measured, and leaf area was calculated for each tissue type. Percent growth was calculated as the area of new growth divided by the total leaf area × 100.
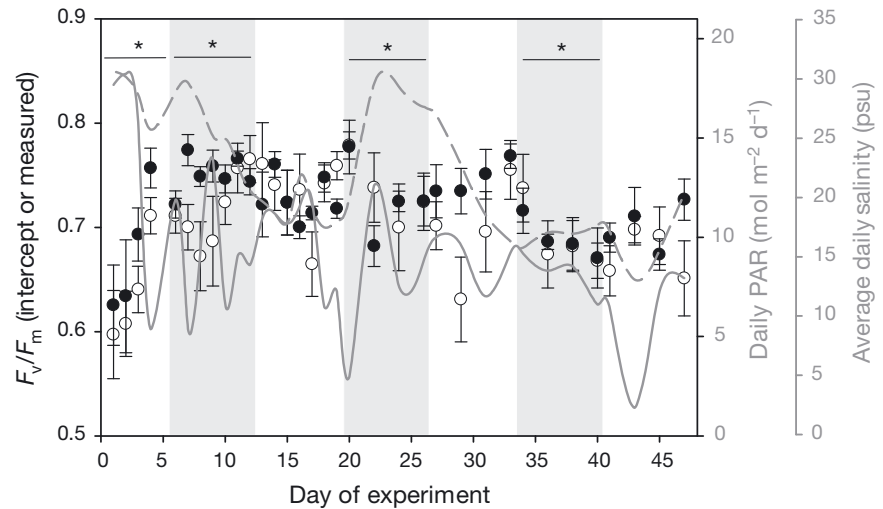
Statistical analysis

Statistical analyses were completed using SAS® and SigmaPlot® for Windows. F_v/F_m (21:00 h), $\Delta F/F_m'$ and PAR values used for each time period (06:00, 09:00, 12:00, 15:00 and 18:00 h) were the average of the 4 shoots sampled from each replicate mesocosm (n = 4). Model I regressions of $\Delta F/F_m'$ vs. PAR were calculated for each day and treatment, and data were pooled for each period (shading and recovery) to calculate averages for each cycle (Cycle 1, Cycle 2, Cycle 3). Parameter estimates from the diurnal regressions of $\Delta F/F_m'$ vs. PAR were compared using a repeated measures 2-way ANOVA (SAS 9.1) with time and treatment as fixed effects. Post-hoc Tukey tests for pairwise comparisons were calculated when p < 0.05. Levenes test was used to assess the assumption of homogeneity of variance. Regression slopes were compared within and between treatments to determine photoacclimation using an ANCOVA calculated in Microsoft Excel. Mean Sun treatment diurnal regressions were compared to mean Shade treatments each day, once a treatment period began.

Variation in leaf morphometric parameters and chlorophyll content among pre-treatment, final Sun and final Shade were compared using 1-way ANOVA on ranks. Variations in leaf area and leaf growth between treatments and within periods (shading and recovery) were compared using 1-way ANOVA on ranks. Tukey's test or Dunn's method (used if treatment group sizes were unequal) was performed for a pairwise multiple comparison when p < 0.05.

RESULTS

The initial 3 d of pre-treatment were sunny and dry; however, unusually frequent rain storms and significantly above-normal total monthly rainfall dominated the remaining duration of this mesocosm experiment (29.6 cm during June 2013 compared with a normal average of 13.2 cm; National Weather Service 2013). This reduced ambient light so that

Fig. 1. Sun treatment maximum photochemical efficiency of PSII (F_v/F_m) (●), effective photochemical efficiency ($\Delta F/F_m'$) vs. photosynthetically active radiation (PAR) regression intercepts (○), daily PAR (solid gray line) and average daily salinity (dashed gray line) vs. day in the experiment. Shading periods are marked by gray areas. *Significant differences (p < 0.05) between F_v/F_m and regression intercepts within periods. n = 4; ±SE
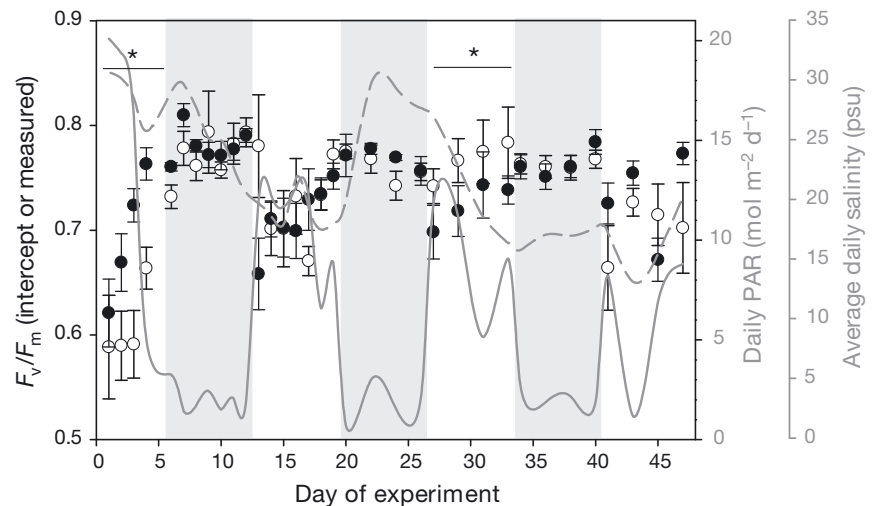
daily PAR never exceeded 13.9 mol m⁻² d⁻¹ after pretreatment, averaging just 8.6 mol m⁻² d⁻¹ for the duration of the Sun (control) treatment. Additionally, the high rainfall caused reductions in salinity for much of the experiment; mesocosm salinities averaged 21.2 for the experiment, with a maximum salinity of 29.9, which is below the normal average salinity for this location (32.2 for January 1, 2009 through December 31, 2012; National Estuarine Research Reserve System 2013).

Chlorophyll fluorescence

Daily PAR exhibited a decreasing trend over the duration of the experiment (Figs. 1 & 2). PAR at the canopy level in the Sun (control) treatment exhibited significant daily variation in response to the frequent rain events and there was also high light attenuation in the mesocosms due to turbid water from runoff. Of the 3 parameters of photosynthetic

health measured (F_v/F_m, $\Delta F/F_m'$ vs. PAR regression slopes and intercepts), slopes showed the highest sensitivity to daily PAR within each cycle and slopes were highly variable from day to day in both treatments (Fig. 3). Regression intercepts (estimates of F_v/F_m) and measured F_v/F_m values were generally similar to each other for both treatments (Figs. 1 & 2). Both F_v/F_m values were higher during shading periods and lower during recovery periods in the Shade treatment. For the first and second cycles of the Shade treatment, estimated and measured F_v/F_m values decreased within 1 to 2 d after shades were removed (after the shading period), reflecting a rapid decrease in photochemical efficiency. This was followed by steady, multi-day increases in F_v/F_m during the recovery period, suggesting photoacclimation and increased photochemical efficiency. Estimated and measured F_v/F_m during the third cycle also showed a rapid decrease with shade removal; however, they did not recover as quickly during the final unshaded period compared to the

Fig. 2. Shade treatment maximum photochemical efficiency of PSII (F_v/F_m) (●), effective photochemical efficiency ($\Delta F/F_m'$) vs. photosynthetically active radiation (PAR) regression intercepts (○), daily PAR (solid gray line) and average daily salinity (dashed gray line) vs. day in the experiment. Shading periods are marked by gray areas. *Significant differences (p < 0.05) between F_v/F_m and regression intercepts within periods. n = 4; ± SE
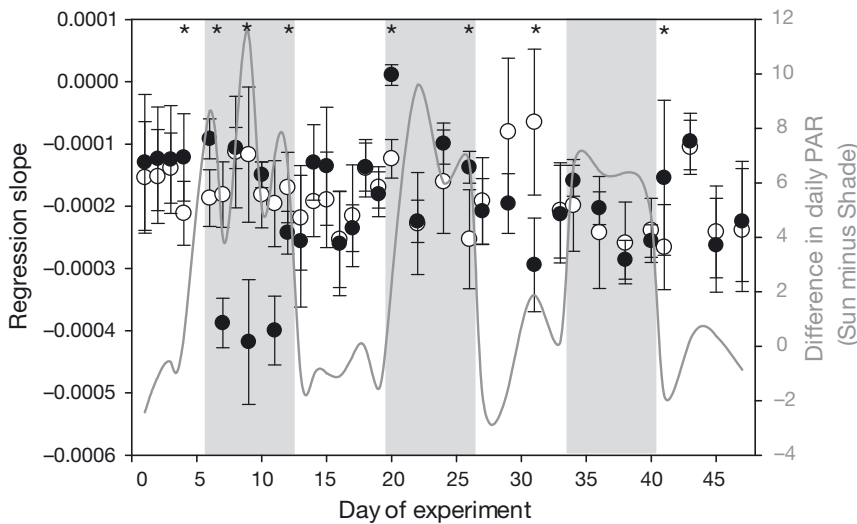
Fig. 3. Regression slopes for diurnal effective photochemical efficiency of PSII ($\Delta F/F_{m}'$) vs. photosynthetically active radiation (PAR) Sun (O) and Shade (●) treatments. Gray line shows differences in daily PAR received between the treatments (scale on right axis). Shading periods are marked by gray areas. *Significant differences ($p < 0.05$) between treatment slopes. n = 4; ± SE

2 previous recovery periods. Sun treatment measured and estimated F_{v}/F_{m} values exhibited significant day-to-day variations reflective of high day-to-day variability in salinity and daily PAR; both showed similar temporal patterns but significantly differed from each other during periods of highly variable salinities or low salinity with moderate variation in daily PAR. Shade treatment estimated and measured F_{v}/F_{m} values were only significantly different to each other during the second recovery period, when salinity was rapidly declining and daily PAR was extremely variable due to the severity of summer rainstorms at this time. Measured and estimated F_{v}/F_{m} values were not significantly different from each other during all shaded periods and during the first and third recovery periods, when

daily PAR levels were relatively stable (first) or low (third). Both treatment F_{v}/F_{m} values and estimates were significantly different during pre-treatment, which corresponded to the largest decrease in daily PAR following the change from clear skies to the initial onset of summer rainstorms.

Treatment-cycle averaged measured or intercept-derived estimates of F_{v}/F_{m}, by period, were generally higher during shading than recovery periods (Fig. 4). Measured F_{v}/F_{m} values were not significantly different among the 3 shading periods or among the 3 recovery periods for the Shade treatment. Shading and recovery periods showed a trend of converging measured F_{v}/F_{m} values over the duration of the experiment, suggesting photoacclimation to the cycle-average daily PAR. Measured
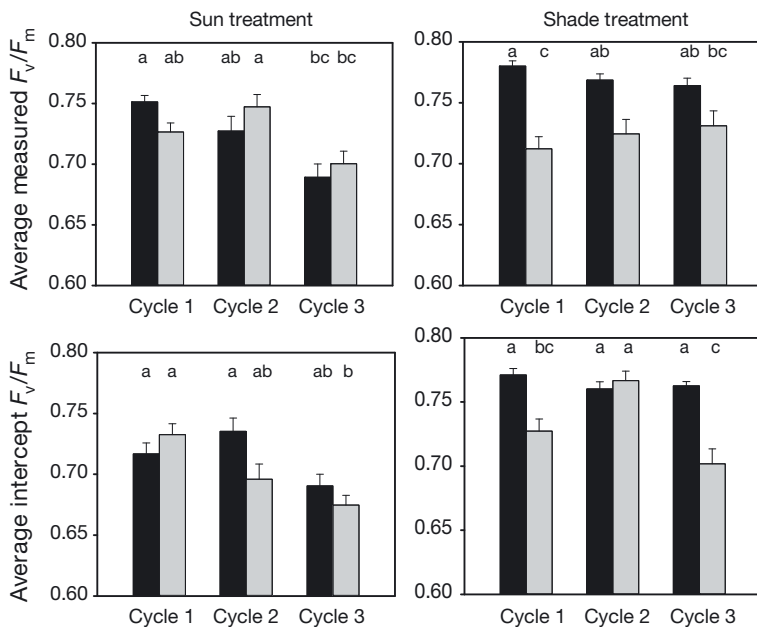

Fig. 4. Relationship of measured dark-acclimated maximum photochemical efficiency of PSII (F_{v}/F_{m}) averaged by treatment period (top) and estimated F_{v}/F_{m} calculated from the intercept of regressions of effective photochemical efficiency of PSII ($\Delta F/F_{m}'$) vs. photosynthetically active radiation (PAR) averaged by treatment period (bottom) for Sun treatment (left) and Shade treatment (right) for the 3 treatment/recovery cycles. Black: shading period; light gray: recovery period; data points: mean ± SE; Cycle 1: n = 28; Cycles 2 and 3: n = 16. Different letters indicate significant differences among treatment period means ($p < 0.05$)

Table 1. Multiple day-to-day comparisons among the last day of one treatment period and the next several days of the following treatment period within each of the 3 treatment cycles for measured maximum photochemical efficiency of PSII (F_v/F_m) and estimated F_v/F_m calculated from the intercepts of daily regressions of effective photochemical efficiency of PSII ($\Delta F/F_m'$) vs. photosynthetically active radiation (PAR) for Sun and Shade treatments. Shaded periods for the Shade treatments are indicated by gray boxes. Uppercase letters indicate significant day-to-day differences (p < 0.05) within a treatment period for the Sun treatment. Lowercase letters indicate significant day-to-day differences within a treatment period for the Shade treatment. Cycle 1: n = 28; Cycles 2 and 3: n = 16

		Cycle 1		Cycle 2		Cycle 3	
		Shading	Recovery	Shading	Recovery	Shading	Recovery
Measured	Sun	A	A	AB A B AB	A	A AB B	A
	Day	4 6 7 8 9 10 11 12	12 13 14 15 16 17 18 19	19 20 22 24 26	26 27 29 31 33	33 34 36 38 40	40 41 43 45 47
	Shade	a	a b ab	a	a	a	a ab a b a
Intercept	Sun	AB B AB A	A AB B AB A	AB A AB B A	AB B C B A	A AB C BC C	A
	Day	4 6 7 8 9 10 11 12	12 13 14 15 16 17 18 19	19 20 22 24 26	26 27 29 31 33	33 34 36 38 40	40 41 43 45 47
	Shade	c ab a ab a	a ab bc abc c abc	a	a	a	a b ab

F_v/F_m exhibited the largest down-regulation after the shades were removed during the first cycle; then the difference between shaded and recovery values decreased during the more overcast second and third cycles. Additionally, measured F_v/F_m values for the third cycle were not significantly different between periods for both treatments; this was a chronically rainy cycle with persistently low irradiance. As expected, Sun treatment estimated and measured F_v/F_m values were not significantly different within cycles, but both exhibited significant declines by the third cycle, suggesting plant stress at the end of the experiment. Shade treatment estimated F_v/F_m indicated significant down-regulation in the first and third cycles, after shades were removed, but they did not decrease during the second recovery period, when salinities exhibited the largest decline. Like the Sun treatment, estimated F_v/F_m values were significantly lower during the final recovery period in the Shade treatment, again suggesting plant stress. Day-wise comparisons between the last day of one period and the next several days of the following period (i.e. shaded to full sun vs. full sun to shaded) within each cycle for estimated and measured F_v/F_m values indicated significant day-to-day variation reflective of both the change in the Shade treatment (shades on or off) and high variability in ambient PAR and salinity (Sun treatment) during the experimental period (Table 1).

Leaf characteristics

Leaf length exhibited a significant decline between pre-treatment and the final recovery period in the Shade treatment (p = 0.019; Table 2). There were no other significant treatment effects on average number of blades per shoot, leaf width, leaf area or leaf dry weight compared to pre-treatment (Table 2). There was no significant difference between treatments for all parameters; however, compared to the Sun treatment, the Shade treatment plants exhibited 26% shorter leaves, a 10% reduction in leaf width, with a 43% decrease in leaf area and 50% decrease in weight (Table 2). The reductions in shoot size in

Table 2. Mean (±SE) for initial and final leaf length (cm), leaf width (cm), leaf area (cm^2), leaf number per shoot and leaf dry weight (g) per shoot for both treatments from morphometric data. Initial: n = 8; final Sun and Shade: n = 4

Parameter	Treatment		
	Initial	Final Sun	Final Shade
Leaf length	12.69 (1.70)	7.53 (1.36)	5.6* (0.62)
Leaf width	0.77 (0.10)	0.53 (0.53)	0.48 (0.06)
Leaf area	29.09 (8.19)	9.75 (3.82)	6.18 (1.30)
Leaf number	2.625 (0.26)	2.25 (0.48)	2.25 (0.25)
Leaf dry weight	0.16 (0.04)	0.06 (0.02)	0.03 (0.005)

*Final treatment measurements that are significantly different from the initial measurement (p < 0.05)

Table 3. Mean ($\pm$ SE) and new growth (%) for leaf length (cm), leaf width (cm), leaf area (cm^2), new leaf area (%), leaf growth rate (cm d^{-1}), leaf number per shoot and leaf dry weight (g) per shoot by period for the Shade treatment from leaf punch. Superscript letters represent final values that are significantly different among periods within treatment ($p < 0.05$). n = 4

Parameter	Cycle 1	Cycle 2		Cycle 3	
	Shading	Shading	Recovery	Shading	Recovery
Leaf length	13.42[a] (3.62)	8.70[ab] (1.54)	6.28[bc] (0.92)	6.28[bc] (0.92)	6.45[bc] (1.27)
New length	13.30 (5.36)	11.63 (4.33)	6.04 (3.49)	28.26 (23.97)	9.24 (4.09)
Leaf width	1.25 (0.28)	0.98 (0.23)	0.93 (0.23)	0.93 (0.23)	1.08 (0.11)
Leaf area	16.82[a] (5.94)	8.52[ab] (2.17)	6.35[bc] (1.37)	5.22[bc] (0.73)	7.44[ab] (2.55)
New leaf area	13.78 (5.26)	11.63 (4.33)	8.35 (5.00)	6.04 (3.49)	9.24 (4.09)
Leaf growth rate	0.56[a] (0.16)	0.26[ab] (0.12)	0.10[b] (0.06)	0.10[b] (0.06)	0.21[ab] (0.09)
Leaf number	2.5 (0.29)	2 (0.41)	1.5 (0.29)	2 (0.41)	2.25 (0.25)
Leaf dry weight	0.11 (0.04)	0.06 (0.01)	0.04 (0.01)	0.03 (0.01)	0.05 (0.02)

both treatments suggest that all shoots were stressed by the end of the experiment.

For all growth parameters (leaf length, percentage growth from leaf length, leaf width, leaf area, percentage growth from leaf area, leaf growth rate, leaf number and total weight) there were no significant differences between treatments within each period (Table 3). Both treatments showed an overall decrease in leaf area and increase in necrotic tissue over time. However, the increase in average leaf number and stable leaf growth rate from Cycle 2 to Cycle 3 indicates that the shoots were still producing new leaves (Table 3). After the final recovery period, the Shade treatment had nearly 50% necrotic leaf tissue, whereas the Sun treatment exhibited less than 40% necrotic tissue. Although shoots in both treatments had green leaves, older blades (Rank 3) frequently separated from the sheath during PAM measurements later in the experiment. The Sun treatment exhibited 37% mortality, whereas the Shade treatment exhibited only 12% mortality over the duration of the experiment. The Sun treatment shoots showed very little change in chlorophyll a (1.36 $\pm$ 0.32 vs. 1.17 $\pm$ 0.21 µg g^{-1} FW), chlorophyll b (0.37 $\pm$ 0.10 vs. 0.34 $\pm$ 0.07 µg g^{-1} FW), or chlorophyll $a{:}b$ ratio (4.01 $\pm$ 0.45 vs. 4.13 $\pm$ 0.78) from the pretreatment to final recovery period. In contrast, both chlorophyll a (2.01 $\pm$ 0.32 µg g^{-1} FW) and chlorophyll b (0.62 $\pm$ 0.09 µg g^{-1} FW) increased, while the chlorophyll $a{:}b$ ratio decreased (3.36 $\pm$ 0.21) in the Shade-treatment leaves.

DISCUSSION

In this mesocosm study, which imposed multiple shading and recovery events as well as unplanned variations in ambient irradiance and salinity, *Thalas-* *sia testudinum* shoots exhibited day-to-day variation in measured and diurnal regression-derived estimates of photosynthetic chemical efficiencies of PSII that frequently equaled or exceeded treatment-induced responses. Regression slopes exhibited the highest day-to-day variation within each treatment period as well as between treatments in response to variations in irradiance and salinity. Durako (2012) suggested that slopes from diurnal $\Delta F/F_m'$ vs. PAR regressions may reflect the efficiency of dissipation of excess energy (photoprotection) or PSII inactivation (photodamage). However, the high sensitivity of this regression-derived parameter to short-term fluctuations in environmental parameters observed in this study suggests it may be of limited use as a reliable indicator of physiological condition in this seagrass. Intercepts of diurnal $\Delta F/F_m'$ vs. PAR regressions and directly measured F_v/F_m showed lower variation within treatment periods than regression slopes. Rapid photoacclimation (1 to 3 d) and more efficient light use were evidenced by increased intercept and F_v/F_m values during periods of reduced irradiance from shading or cloudy weather. With an increase in available irradiance following shade removal, *T. testudinum* shoots exhibited a short-term (1 to 2 d) decline in photochemical efficiency, indicative of down-regulation and photoprotection, followed by a longer-term (3 to 4 d) up-regulation of photochemical efficiency, suggesting photoacclimation.

High day-to-day variations in photochemical efficiency in response to overcast or hyposaline conditions indicate that *T. testudinum* exhibits rapid photoacclimatory reponses to short-term weather changes and suggests that PAM fluorometric measurements may not be the most effective method for assessing seagrass physiological health under variable field conditions. Longstaff et al. (2002) com-

pared diurnal variations in photosynthetic oxygen exchange and PAM fluorescence in the marine chlorophyte macroalgae *Ulva lactuca* and concluded that PAM fluorescence was a good indicator of photosynthetic rate at only low-to-medium irradiances, because of increases in electron cycling in PSII or non-photochemical quenching at higher irradiances. Levy et al. (2004) compared oxygen evolution and $\Delta F/F_m'$ in 5 coral species and reported that $\Delta F/F_m'$ exhibited higher variability and diurnal hysteresis patterns often opposite to those of oxygen. They concluded that diurnal hysteresis in chlorophyll fluorescence may vary among and within species because of microscale variability in bio-optical properties in the coral colonies. Schubert et al. (2015) also observed significant diurnal variability in F_v/F_m in *T. testudinum* under high light conditions, which they attributed to a heterogeneous leaf photoacclimatory condition to minimize photodamage in the upper canopy while maximizing productivity in the lower shaded portions of the canopy.

Diurnal $\Delta F/F_m'$ vs. PAR regression intercepts and measured F_v/F_m did exhibit similar response patterns to environmental changes in this study, supporting previous suggestions (Durako 2012, Howarth & Durako 2013a) that diurnal regression intercepts may serve as a proxy for F_v/F_m for *T. testudinum* when direct dark-acclimated measurements are not feasible. Estimated F_v/F_m values were significantly different from measured F_v/F_m, mainly during periods combining stressors of rapid declines in irradiance and salinity. This pattern of responses for estimated and measured F_v/F_m, being similar under non-stressful conditions but differing when stressed has previously been reported for *T. testudinum* seedlings (Howarth & Durako 2013a). Shoots in the ambient light (Sun) treatment also showed significant differences between estimated and measured F_v/F_m only when environmental conditions became stressful (high rainfall with highly variable salinity). This pattern may reflect differing sensitivities of PSII to light and salinity stress under variable light pressure (State 1) vs. under stable dark conditions (State 2; Papageorgiou & Stamatakis 2004).

Consistent with previous reports, Shade treatment shoots generally displayed higher photochemical efficiencies when shaded and the increasing F_v/F_m values in the third recovery period indicate a shift to a more low light photoacclimation state in these shoots (Major & Dunton 2002, Durako et al. 2003, Cayabyab & Enríquez 2007, Ochieng et al. 2010). The Shade shoots had a higher F_v/F_m ratio and a greater decrease in leaf area and leaf weight com-

pared to the Sun plants, suggesting that these plants were able to photoacclimate but were not thriving. Although the final recovery period in the Shade treatment did not result in the same rapid responses in photochemical efficiencies as the first 2 recovery periods, this was most likely due to stress, related to the continued low irradiance levels following shade removal because of heavy rainfall during this final unshaded period. Decreasing photochemical efficiency, increased necrotic tissue and higher mortality indicates that even the Sun plants were stressed by the final recovery period.

Shading intensity and duration can affect the timeline and magnitude of seagrass responses (Lavery et al. 2009, Collier et al. 2012). Morphological and pigment changes in the *T. testudinum* shoots at the end of the experiment were consistent with the treatment cycle-averaged PAM chlorophyll fluorescence results (Fig. 4) indicating light limitation (Marquardt et al. 2010). With relatively low ratio of above-ground to below-ground biomass (~15%), *T. testudinum* has high light requirements (Fourqurean & Zieman 1991). Significant decreases in leaf elongation rate from leaf-punch data were detected after the second shading period, despite no overall significant decreases in leaf morphometrics between treatments. However, the additional light available in the Sun treatment (control) resulted in greater leaf size and shoot biomass compared to the Shade shoots (Table 1). Changes in leaf chlorophyll concentrations, F_v/F_m and leaf senescence were observed in both treatments as indicators of stress. Increases in the Shade shoots treatment cycle-averaged F_v/F_m corresponded with increases in leaf chlorophyll *a* and *b* contents and a reduction in *a*:*b* ratios (low light acclimated), which are consistent with previous shade responses (Major & Dunton 2002, Cayabyab & Enríquez 2007, Howarth & Durako 2013b). Shaded shoots produced more chlorophyll *a* and *b*, which may increase capture of light at longer wavelengths, predominant under low light conditions (Lee & Dunton 1997, Ralph et al. 2007, Biber et al. 2009). However, under chronic light deprivation, reductions in pigment concentration and carbon reserves may also occur as the plant attempts to maintain a positive balance between energy input and metabolic demands (Touchette & Burkholder 2000). These response strategies to chronic light limitation allow plants to tolerate low light conditions more efficiently (Grubb 1998, Collier et al. 2012).

During this mesocosm study, the *T. testudinum* shoots were exposed to a large number of simultaneous influences (chronic reductions in irradiance and

large changes in salinity) that were not part of the original experimental design. After the first shading period, F_v/F_m values returned to pre-shade values within 3 d; however, with repeated shading and chronic low light levels from overcast and rain, plants in the Shade treatment exhibited more persistent decreases in yields. The reduction in both ambient irradiance and salinity later in this study may have exacerbated the intensity of the shading treatment and reduced recovery responses (Lavery et al. 2009). Nevertheless, the Shade treatment plants maintained F_v/F_m values >0.6 even at the end of the experiment, which indicates that while stressed, they were still photosynthetically active with minimal PSII inactivation. *T. testudinum* may recover from short-term hyposaline (20) exposure if conditions are returned to their physiological tolerance range of 20 to 40 (Fernandez-Torquemada & Sanchez-Lizaso 2005, Kahn & Durako 2006, Howarth & Durako 2013b). Our salinity and irradiance variations were reflective of conditions that can occur in Florida Bay during wet summers, and the shoots showed rapid responses in chlorophyll fluorescence to repeated shading and to day-to-day variations in light and salinity. PAM fluorescence has been shown to be a good indicator of salinity stress if measured under uniform PAR (Pages et al. 2010, Howarth & Durako 2013a, Olivé et al. 2013), but our results indicate that it may not be possible to extrapolate single-treatment laboratory data to highly variable field conditions (Romero et al. 2007, Gera et al. 2012).

PAM fluorometry is an attractive physiological assessment method as it allows rapid, multiple, non-invasive field measurements with little to no damage or stress to the plant (Gera et al. 2012). However, it may not be a robust ecological bioindicator, as sea-grass photophysiological processes have not always responded consistently to light reduction (McMahon et al. 2013). Previous light history is extremely important when assessing diurnal and day-to-day variation in seagrass photosynthetic responses (Ralph et al. 1998, Durako & Kunzelman 2002, Belshe et al. 2007, 2008). Our *T. testudinum* shoots exhibited significantly increased efficiency of photochemical processes following the first shading, suggesting they were becoming shade-acclimated within 1 to 3 d, but they exhibited almost immediate reductions in estimated and measured F_v/F_m values when shades were removed. Our results confirm previous observations that *T. testudinum* shoots exhibit rapid physiological plasticity to different light conditions, which needs to be considered when interpreting day-to-day variations in photosynthetic response (Durako &

Kunzelman 2002, Belshe et al. 2007, 2008, Cayabyab & Enríquez 2007). Our results additionally suggest that daily variations in weather, such as those occurring during the rainy season in Florida Bay, may restrict the application of landscape-scale PAM-derived assessments of physiological condition in this species.

LITERATURE CITED

Beer S, Björk M (2000) Measuring rates of photosynthesis of two tropical seagrasses by pulse amplitude modulated (PAM) fluorometry. Aquat Bot 66:69–76

Beer S, Vilenkin B, Weil A, Veste M, Susel L, Eshel A (1998) Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. Mar Ecol Prog Ser 174:293–300

Beer S, Björk M, Gademann R, Ralph PJ (2001) Measurement of photosynthesis in seagrasses. In: Short FT, Coles R (eds) Global seagrass research methods. Elsevier, Amsterdam, p 183–198

Belshe EF, Durako MJ, Blum JE (2007) Photosynthetic rapid light curves (RLC) of *Thalassia testudinum* exhibit diurnal variation. J Exp Mar Biol Ecol 342:253–268

Belshe EF, Durako MJ, Blum JE (2008) Diurnal light curves and landscape-scale variation in photosynthetic characteristics of *Thalassia testudinum* in Florida Bay. Aquat Bot 89:16–22

Biber PD, Kenworthy WJ, Paerl HW (2009) Experimental analysis of the response and recovery of *Zostera marina* (L.) and *Halodule wrightii* (Ascher.) to repeated light-limited stress. J Exp Mar Biol Ecol 369:110–117

Butler MJ IV, Hunt JH, Herrnkind WF, Childress MJ and others (1995) Cascading disturbances in Florida Bay USA: cyanobacteria blooms, sponge mortality, and implications for juvenile spiny lobsters *Panulirus argus.* Mar Ecol Prog Ser 129:119–125

Cayabyab NM, Enríquez S (2007) Leaf photoacclimatory responses of the tropical seagrass *Thalassia testudinum* under mesocosm conditions: a mechanistic scaling-up study. New Phytol 176:108–123

Collier CJ, Waycott M, Ospina AG (2012) Responses of four Indo-West Pacific seagrass species to shading. Mar Pollut Bull 65:342–354

Durako MJ (2012) Using PAM fluorometry for landscape-level assessment of *Thalassia testudinum*: Can diurnal variation in photochemical efficiency be used as an ecoindicator of seagrass health? Ecol Indic 18:243–251

Durako MJ, Kunzelman JI (2002) Photosynthetic characteristics of *Thalassia testudinum* measured *in-situ* by pulse-amplitude modulated (PAM) fluorometry: methodological and scale-based considerations. Aquat Bot 73:173–185

Durako MJ, Hall MO, Merello M (2002) Patterns of change in the seagrass dominated Florida Bay hydroscape. In: Porter JW, Porter KG (eds) The Everglades, Florida Bay

and coral reefs of the Florida Keys: an ecosystem source-book. CRC Press, Boca Raton, FL, p 523–537

Durako MJ, Kunzelman JI, Kenworthy WJ, Hammerstrom KK (2003) Depth-related variability in the photobiology of *Halophila johnsonii* and *Halophila decipiens.* Mar Biol 142:1219–1228

Enriquez S, Merino M, Iglesias-Prieto R (2002) Variations in the photosynthethetic performance along the leaves of the tropical seagrass *Thalassia testudinum*. Mar Biol 140: 891–900

Falkowski PG, Jokeil PL, Kinzie RA (1990) Irradiance and corals. In: Dubinski Z (ed) Ecosystems of the world—coral reefs. Elsevier, Amsterdam, p 89–107

Fernandez-Torquemada Y, Sanchez-Lizaso JL (2005) Effects of salinity on leaf growth and survival of the Mediterran-ean seagrass *Posidonia oceanica*. (L.) Delile. J Exp Mar Biol Ecol 320:57–63

Fourqurean JW, Zieman JC (1991) Photosynthesis, respira-tion and whole plant carbon budget of the seagrass *Tha-lassia testudinum.* Mar Ecol Prog Ser 69:161–170

Gera A, Alcoverro T, Mascaro O, Perez M, Romero J (2012) Exploring the utility of *Posidonia oceanica* chlorophyll fluorescence as an indicator of water quality within the European Water Framework Directive. Environ Monit Assess 184:3675–3686

Grubb PJ (1998) A reassessessment of the strategies of plants which cope with shortages of resources. Perspect Plant Ecol Evol Syst 1:3–31

Howarth JF, Durako MJ (2013a) Diurnal variation in chloro-phyll fluorescence of *Thalassia testudinum* seedlings in response to controlled salinity and light conditions. Mar Biol 160:591–605

Howarth JF, Durako MJ (2013b) Variation in pigment con-tent of *Thalassia testudinum* seedlings in response to changes in salinity and light. Bot Mar 56:261–273

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a, b, c*$_1$, and *c*$_2$ in higher plants, algae, and natural phytoplankton. Plant Physiol Biochem 167:191–194

Kahn AE, Durako MJ (2006) *Thalassia testudinum* seedling responses to changes in salinity and nitrogen levels. J Exp Mar Biol Ecol 335:1–12

Lavery PS, McMahon K, Mulligan M, Tennyson A (2009) Interactive effects of timing, intensity and duration of experimental shading on *Amphibolis griffithii*. Mar Ecol Prog Ser 394:21–33

Lee KS, Dunton KH (1997) Effects of *in situ* light reduction on the maintenance, growth and partitioning of carbon resources in *Thalassia testudinum* Banks ex König. J Exp Mar Biol Ecol 210:53–73

Levy O, Dubinsky Z, Schneider K, Achituv Y, Zakai D, Gor-bunov MY (2004) Diurnal hysteresis in coral photosyn-thesis. Mar Ecol Prog Ser 268:105–117

Longstaff BJ, Kildea T, Runcie JW, Cheshire A and others (2002) An *in situ* study of photosynthetic oxygen exchange and electron transport rate in the marine macroalga *Ulva lactuca* (Chlorophyta). Photosynth Res 74:281–293

Madden CJ, Rudnick DT, McDonald AA, Cunniff KM, Fourqurean JW (2009) Ecological indicators for assessing and communicating seagrass status and trends in Florida Bay. Ecol Indic 9(Suppl):S68–S82

Major KM, Dunton KH (2002) Variations in light harvesting characteristics of seagrass, *Thalassia testudinum*: evi-dence for photoacclimation. J Exp Mar Biol Ecol 275: 173–189

Marquardt S, Schubert H, Varela DA, Huovinen P, Hen-

riquez L, Buschmann AH (2010) Light acclimation strate-gies of three commercially important red algal species. Aquaculture 299:140–148

McMahon K, Collier C, Lavery PS (2013) Identifying robust bioindicators of light stress in seagrasses: a meta-analy-sis. Ecol Indic 30:7–15

National Estuarine Research Reserve System (2013) System-wide monitoring program. Available at: www.nerrsdata. org/ (accessed October 14–25, 2013)

National Weather Service (2013) Supplemental rainfall data 1871–2013. Available at: www.erh.noaa.gov (accessed October 14–25, 2013)

Ochieng CA, Short FT, Walker DI (2010) Photosynthetic and morphological response of eelgrass (*Zostera marina* L.) to a gradient of light conditions. J Exp Mar Biol Ecol 382: 117–124

Olivé I, Vergara JJ, Pérez-Llorens JL (2013) Photosynthetic and morphological photoacclimation of the seagrass *Cymodocea nodosa* to season, depth and leaf position. Mar Biol 160:285–297

Pages JF, Perez M, Romero J (2010) Sensitivity of the sea-grass *Cymodocea nodosa* to hypersaline conditions: a microcosm approach. J Exp Mar Biol Ecol 386:34–38

Papageorgiou GC, Stamatakis K (2004) Water and solute transport in cyanobacteria as probed by chlorophyll fluo-rescence. In: Papageorgiou GC, Govindjee (eds) Chloro-phyll *a* fluorescence: a signature of photosynthesis. Springer, Dordrecht, p 663–678

Ralph PJ, Gademann R (2005) Rapid light curves: a powerful tool for the assessment of photosynthetic activity. Aquat Bot 82:222–237

Ralph PJ, Gademann R, Dennision WC (1998) *In-situ* sea-grass photosynthesis measured using a submersible, pulse-amplitude modulated fluorometer. Mar Biol 132: 367–373

Ralph PJ, Durako MJ, Enríquez S, Collier CJ, Doblin MA (2007) Impact of light limitation on seagrasses. J Exp Mar Biol Ecol 350:176–193

Robblee MB, Barber TR, Carlson PR, Durako MJ and others (1991) Mass mortality of the tropical seagrass *Thalassia testudinum* in Florida Bay (USA). Mar Ecol Prog Ser 71: 297–299

Romero J, Martinez-Crego B, Alcoverro T, Perez M (2007) Multivariate index based on the seagrass *Posidonia oceanica* (POMI) to assess ecological status of coastal waters under the framework directive (WFD). Mar Pollut Bull 55:196–204

Schreiber U, Schliwa U, Bilger W (1986) Continuous record-ing of photochemical and non-photochemical chloro-phyll fluorescence quenching with a new type of modu-lation fluorometer. Photosynth Res 10:51–62

Schubert N, Colombo-Pallota MF, Enríquez S (2015) Leaf and canopy scale characterization of the photoprotective response to high-light stress of the seagrass *Thalassia testudinum.* Limnol Oceanogr 60:286–302

Silva J, Sharon Y, Santos R, Beer S (2009) Measuring sea-grass photosynthesis: methods and applications. Aquat Biol 7:127–141

Touchette BW, Burkholder JM (2000) Overview of the phys-iological ecology of carbon metabolism in seagrasses. J Exp Mar Biol Ecol 250:169–205

Zieman JC (1974) Methods for the study of the growth and productivity of turtle grass, *Thalassia testudinum* König. Aquaculture 4:139–143

Zieman JC (1982) The ecology of the seagrasses of South Florida: a community profile. US Fish and Wildlife Serv-ices, Washington, DC