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Experimental analysis of the response and recovery of *Zostera marina* (L.) and *Halodule wrightii* (Ascher.) to repeated light-limitation stress

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ABSTRACT

Seagrasses are considered important indicators of decline in water quality resulting in increased light attenuation that negatively influences their growth and survival. Chronic light-limitation interspersed with unpredictable acute attenuation events have had poorly understood effects on seagrass recovery dynamics. *Zostera marina* (eelgrass) and *Halodule wrightii* (shoalgrass) were subject to a matrix of light-deprivation events followed by recovery periods to mimic repeated acute shading events. Plant survival, morphology, biomass, chlorophyll content, and Fv/Fm were assessed over time to determine recovery. At the end of the experiment, all plants were harvested and species-specific treatment effects were determined. Significant differences due to treatments were noted in all parameters measured. In general, responses were similar for both life-stages and between species, suggesting similar physiological tolerance to repeated acute light-attenuation events. Only plants in treatments where light-deprivation was followed by a recovery interval of at least the same duration showed signs of long-term survival. Chlorophyll fluorescence (Fv/Fm) was an important metric for assessing recovery, but it failed to detect the onset of mortality in many plants. Other metrics of plant condition need to be assessed and coupled with chlorophyll fluorescence data to assess seagrass "health". This is of particular importance in field studies, where the history of the plants is largely unknown.

1. Introduction

Seagrasses are important marine benthic primary producers that play a central role in the stability, nursery function, biogeochemical cycling, and trophodynamics of coastal ecosystems (Hemminga and Duarte, 2000; Larkum et al., 2006). Seagrass meadows stabilize sediments, which would otherwise be easily resuspended and result in increased and prolonged turbidity reducing benthic primary production (Moore et al., 1997; Koch, 2001). Water clarity is therefore an important abiotic factor determining distribution, abundance, and survival of seagrasses, in large part because of their high light requirements (Duarte ,1991; Batiuk et al., 2000; Dixon, 2000). Of particular interest to seagrass survival are system-wide changes that can cause chronic light limitation (e.g., increased phytoplankton or macroalgal abundance) or event–driven acute light attenuation (e.g., storm mediated sediment suspension or runoff-derived turbidity plumes).

Acute light limitation due to elevated turbidity occurs as sediment inputs to coastal waters have increased with altered land-use patterns, while dredging, boating activities, and shoreline hardening also contribute to increased sediment resuspension (Koch, 2001; Ralph et al., 2007). Pulsed light limitation events can also arise from phytoplankton or drifting macroalgal blooms, symptomatic of eutrophication, especially in systems with long water residence times (Smith et al., 1999; Hauxwell, et al., 2006). The underwater light environment varies substantially as a function of wave refraction, clouds, tidal amplitude, climate, and anthropogenic effects including nutrient and sediment inputs, which all affect water-quality and light availability (Gallegos, 1994; Kirk, 1994; Falkowski and Raven, 2007; Ralph et al., 2007). For example, in the North River, N.C., three day acute light-attenuation events occurred 1-3 times per month in data collected over 2 years of continuous photosynthetically active radiation measurements (PAR) and water quality monitoring over seagrass beds (Biber et al., 2005, 2008).

Prior research has focused on understanding the effects of declining water quality associated with eutrophication (Short and Burdick, 1996), and the concomitant reduction in water clarity that can promote seagrass declines (e.g., Dennison et al., 1993; Orth et al., 2006). Determinations of the light requirements of seagrasses *in situ* (Kenworthy and Haunert, 1991; Kenworthy and Fonseca 1996) and in controlled experiments (Dennison and Alberte 1982, 1986; Lee et al., 2007) have contributed to a better understanding of seagrass light-limitation. These experimental and observational approaches have

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Table 1A
Abiotic conditions measured in spring for the *Zostera marina* experiment and in summer for the *Halodule wrightii* experiment. A: Water quality measured in tank 3 with YSI 6000 UPG probe

	Zostera			Halodule		
	min	Mean±S.D.	max	min	Mean±S.D.	max
Temp °C	18.92	20.36±1.4	25.38	24.24	27.62±1.39	33.42
Salinity (ppt)	25.12	28.51±1.46	31.61	29.88	31.96±2.89	38.04
Turbidity (NTU)	4.1	10.75±1.87	16.9	1.2	5.15±2.36	30.4
pH	5.78	7.64±0.52	8.15	6.63	7.78±0.28	8.39

Table 1B
Abiotic conditions measured in spring for the *Zostera marina* experiment and in summer for the *Halodule wrightii* experiment. B: Mean±S.D. PAR (mol m^{-2} day^{-1}) available to seagrass plants in the two experiments and number of hours per day exceeding saturation intensity (H_{sat})

Treatment	*Zostera*	#H_{sat}	*Halodule*	#H_{sat}
L	11.07±3.196	>24	10.89±4.525	9.5
3D:9L	7.69±3.721	21.4	8.53±3.034	7.4
3D:3L	5.41±3.501	15.0	5.70±5.617	4.9
9D:9L	5.49±3.374	15.3	5.66±5.404	4.9
9D:3L	2.58±3.034	7.2	3.36±6.363	2.9
D	0±0	0.0	0±0	0.0

Potential PAR limitation was based on: *Zostera* I_{sat}=0.36 mol m^{-2} hr^{-1}×H_{sat}>9 hrs (Dennison and Alberte, 1982, 1985) and *Halodule* I_{sat}=1.15 mol m^{-2} hr^{-1}×H_{sat}>8 hrs (Dunton and Tomasko, 1994; Dunton, 1994).

typically focused on correspondence analyses or subjecting plants to chronic, long-term light-deprivation while measuring declines of plant vigor (Ralph and Burchett, 1995; Ralph, 1999a,b). Very few studies have looked at the potential for recovery from such stress (Longstaff and Dennison, 1999; Longstaff et al., 1999; Bite et al., 2007), and none to date have examined how repeated stress-recovery intervals affect the long-term viability of individuals and different life stages. As the light-limited loss of seagrass plants takes place most often at the deep-edge, a better understanding of the tolerance of those chronically light-limited plants to additional acute light-attenuation events will contribute to predicting seagrass distribution. Their capability to recover from such events becomes critical for predicting future responses of the meadow and is important for managing seagrass resources.

The aim of this study was to investigate the responses of *Zostera marina* (eelgrass) and *Halodule wrightii* (shoalgrass) during and after repeated periods of total light deprivation (i.e., total darkness). Total light deprivation was used to simulate adverse pulsed conditions of extreme light-limitation due to reduced water clarity (e.g., turbidity plume). The specific objectives of the study were to measure changes in (i) survival rate, (ii) morphological attributes, (iii) leaf chlorophyll a, and (iv) chlorophyll a fluorescence following repeated periods of total light deprivation.

2. Methods

2.1. Experimental design

Seedlings (<1 month old) and mature plants (>1 year old) of *Z. marina* were collected from seagrass beds in the vicinity of Beaufort, NC (34° 43'N, 76° 40'W) in early spring, while mature *H. wrightii* was collected during the summer, because of seasonal changes in the dominant species (Thayer et al., 1977). Plants with intact roots, rhizomes, and shoots were gently removed from the sediments, transported to the lab, cleaned with seawater and planted within less than 24 hours after collection. Seedlings of *Z. marina* and mature individuals of both species were planted in the same manner. Six individuals of the same species and life-stage were planted into a tub (30×35×14 cm) filled with cleaned and sieved beach sand that had been acclimated for >1 month in flow-through seawater tanks. Plants were inserted into the sand to approximately the same depth as the field location from where they were collected. Twelve tubs, containing 72 seagrass plants, were placed in concrete burial crypts (200×75×50 cm) housed in a temperature controlled greenhouse at the Center for Coastal Fisheries and Habitat Research, NOAA, Beaufort, N.C. Experimental treatments were randomly allocated to each tank partition, and the replicate partitions were located on opposite sides of the greenhouse. Plants were then allowed to recover from transplanting stress for 2 weeks prior to commencement of the experiments.

The *Z. marina* experiment began on March 13 and ended April 28, 2004. The *H. wrightii* experiment ran from July 26 to September 6, 2004. Six different light stress-recovery treatments were tested: light control (L), dark control (D), 3 days dark followed by 9 days light (3D:9L) and repeated, 3D:3L, 9D:9L and 9D:3L. The 9 day shading duration was selected as a three-fold increase in stress duration compared to the observed conditions (Biber et al., 2005, 2008). Supplemental lighting was provided by banks of twelve 100 W halogen floodlights placed above each tank about 1 m from the water surface to provide irradiances of 75-100 μE m^{-2} s^{-1} at canopy level; lights were operated on a 14:10 hr (L:D) cycle. Seawater was derived from a nearby tidal channel and delivered to all the tanks through a semi-recirculating system, after allowing particle settling and sand filtration. Water temperature was maintained between 20-25 °C in spring and 25-30 °C in summer, comparable to ambient conditions in North Carolina estuaries.

2.2. Abiotic variables

All abiotic variables were recorded hourly for the duration of the experiment. Temperatures were monitored in each of the 6 tanks and water quality was monitored using a YSI 6000 UPG multi-parameter sonde for temperature, salinity, and turbidity (NTU) in tank 3. Photosynthetically active radiation (PAR) reaching the leaf canopy was obtained using two spherical quantum irradiance sensors (Li-Cor), one each in tank 3 and 5 (the two L treatments), both connected to an LI-1000 data logger. Light data were converted to integrated daily values, averaged over the experimental period, and compared to the daily saturation light requirements for both species.

2.3. Biotic Measurements and Survival

To determine responses to acute light-limitation stress in both seagrass species, the following parameters were assessed: (1) Survival of individuals; (2) Plant morphological attributes; (3) Leaf chlorophyll content; and (4) chlorophyll fluorescence, Fv/Fm. All living plants were analyzed at the beginning and end of the experiment. Plants

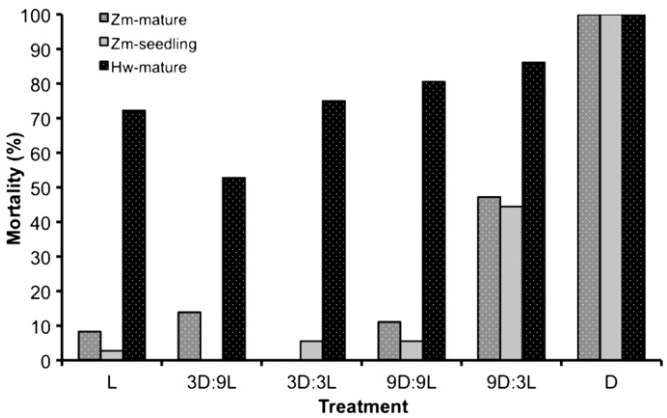

Fig. 1. Mortality by species and stage in the two experiments. Each of the 6 treatments had 36 individuals. Zm=*Zostera marina*, Hw=*Halodule wrightii*. No seedling stage of Hw was available to test.

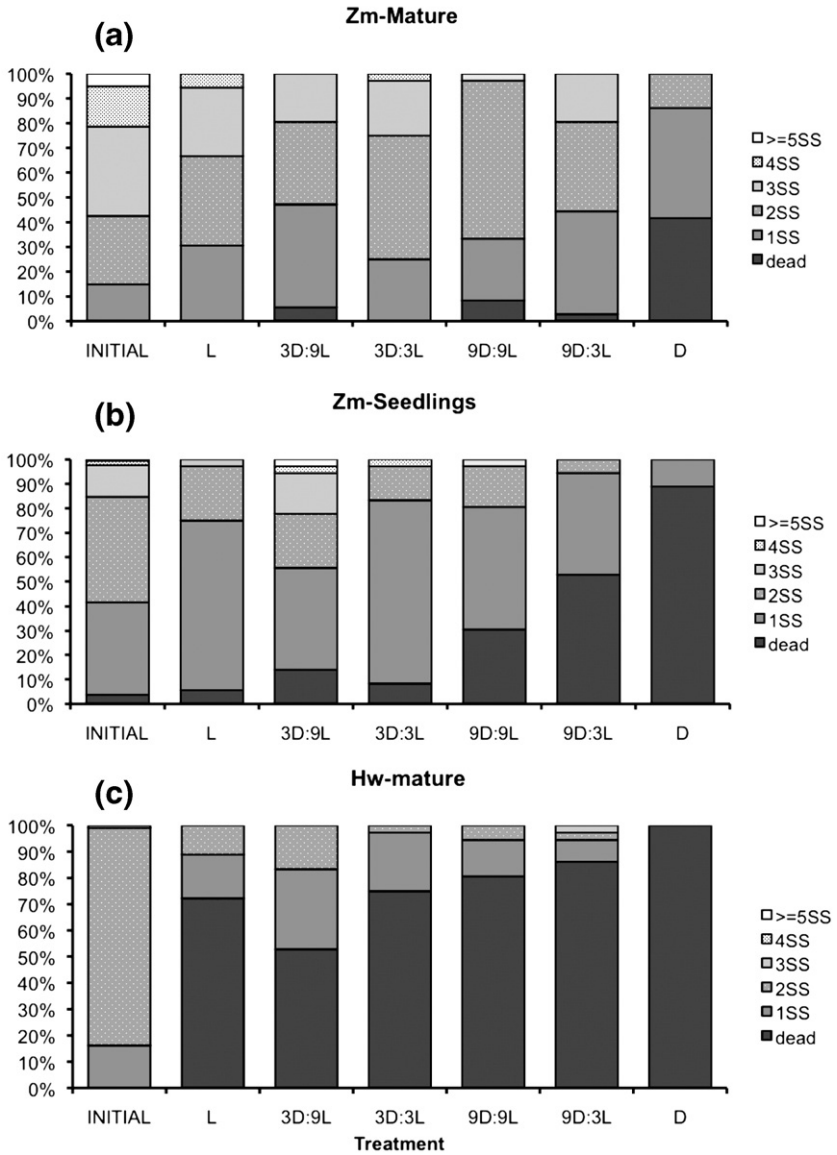

Fig. 2. Percent of population with a given number of standing shoots (SS), ranging from 0 to 5 shoots per plant, found on (a) *Zostera marina* (Zm) mature, (b) seedlings, and (c) *Halodule wrightii* (Hw) plants at the beginning and in each of the 6 treatments at the end of the experiment.

were considered living if leaves appeared green, chlorophyll fluorescence was detected, and they were firmly attached to the rhizome at the base of the individual shoots.

2.4. Morphology

Seedlings of *Z. marina* had one shoot at time of planting, while mature plants of both *Z.marina* and *H. wrightii* had two shoots connected by a common rhizome. The number of shoots, and leaves per shoot, were counted after the 2 week acclimation period and again at end of the experiment. The length of the longest leaf per shoot was measured (nearest mm) from the base of the leaf where it attached to the sheath (usually at the sediment level) up to the intact tip; we noted whether the tip was intact or broken off and only intact leaves were used in calculations. Leaf width of this longest leaf was measured (nearest 0.1 mm); all leaves on a shoot were assumed to be the same width. For all three morphometric variables, the final treatment means for each species or stage were analyzed by one-way ANOVA followed by Tukey's post-hoc test. To meet the assumptions of normality in ANOVA, the number of leaves per shoot variable was log transformed, leaf length was square root transformed, and leaf width was not transformed.

2.5. Biomass

Biomass samples of all surviving plant material was harvested at the end of each experiment, rinsed clean of debris and frozen at −4 °C. After thawing, all leaves in a sample (=treatment) were scraped clean of visible epiphytes with a glass microscope slide. Seagrass roots were free of sediments with tap water. Seagrass tissues were then separated into above-ground (leaf/sheath) and below-ground (root/rhizome) material and dried at 60 °C for 48 hours, before weighing (nearest 0.1 mg).

In order to standardize for differential mortality across the 6 treatments, a subsample of 10 randomly-selected plants per treatment was used in statistical comparisons. The longest leaf was randomly chosen from 5 of the plants. Epiphytes were scraped off the leaf and the five epiphyte-free leaves were used to determine leaf area: dry weight relationships for subsequent calculations of chlorophyll content for each treatment before being added back to the above-ground

Table 2
Morphological attributes (mean ± S.D.) of two seagrasses, *Zostera marina* in mature and seedling life stages and *Halodule wrightii* in mature life stage only

Treatment	# Leaves/ Shoot	Leaf Length (mm)	Leaf Width (mm)
Zostera - Mature			
Initial	5.2 ± 0.11	168.3 ± 3.35	2.19 ± 0.045
Light	4.2 ± 0.21 ^{A}	305.3 ± 22.95 ^{A}	2.24 ± 0.072 ^{A}
3D:9L	4.1 ± 0.34 ^{AB}	251.5 ± 28.20 ^{AB}	2.30 ± 0.072 ^{A}
3D:3L	3.3 ± 0.15 ^{AB}	210.1 ± 10.67 ^{B}	2.06 ± 0.068 ^{A}
9D:9L	3.6 ± 0.33 ^{AB}	141.7 ± 11.73 ^{C}	2.13 ± 0.067 ^{A}
9D:3L	3.0 ± 0.34 ^{B}	142.0 ± 12.59 ^{C}	2.13 ± 0.068 ^{A}
Dark	2.0 ± 0.39 ^{C}	44.7 ± 8.46 ^{D}	1.74 ± 0.112 ^{B}
Zostera - Seedling			
Initial	4.3 ± 0.08	112.4 ± 2.35	1.85 ± 0.032
Light	3.7 ± 0.34 ^{A}	175.7 ± 13.19 ^{A}	2.09 ± 0.059 ^{A}
3D:9L	3.1 ± 0.35 ^{AB}	160.2 ± 18.61 ^{AB}	1.89 ± 0.049 ^{AB}
3D:3L	2.9 ± 0.31 ^{AB}	102.1 ± 9.67 ^{BC}	1.81 ± 0.053 ^{AB}
9D:9L	2.2 ± 0.35 ^{B}	70.0 ± 11.30 ^{C}	1.89 ± 0.101 ^{AB}
9D:3L	1.0 ± 0.21 ^{C}	33.1 ± 7.44 ^{D}	1.59 ± 0.114 ^{B}
Dark	0.2 ± 0.09 ^{D}	3.2 ± 1.60 ^{E}	0.44 ± 0.131 ^{C}
Halodule – Mature			
Initial	0.64 ± 0.018	161.7 ± 4.25	0.94 ± 0.012
Light	0.93 ± 0.067 ^{A}	45.0 ± 11.14 ^{BC}	0.82 ± 0.032 ^{A}
3D:9L	0.90 ± 0.045 ^{A}	73. 5 ± 11.39 ^{A}	0.88 ± 0.048 ^{A}
3D:3L	0.89 ± 0.074 ^{A}	50.2 ± 7.53 ^{B}	0.78 ± 0.022 ^{A}
9D:9L	0.93 ± 0.071 ^{A}	36.4 ± 8.81 ^{BC}	0.85 ± 0.032 ^{A}
9D:3L	0.90 ± 0.010 ^{A}	40.8 ± 7.29 ^{BC}	0.91 ± 0.019 ^{A}
Dark	0 ± 0 ^{B}	0 ± 0 ^{C}	0 ± 0 ^{B}

Sample size was n = 216 initial, and n = 36 per treatment for final measurements. Superscripts are means that were not significantly different from one another by Tukey's HSD post-hoc comparison.

biomass sample. For both biomass variables, the final treatment means for each species/stage were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Prior to analyses, above- and below-ground biomass were log transformed for both species.

2.6. Leaf Chlorophyll

Leaf tissue samples were collected from 6 leaves per treatment at the end of the trial and frozen at −20 °C. Duplicate leaf samples from the mid-section of a leaf, where Fv/Fm had been measured (see below), were ground in a pestle and mortar (<6months after harvest). Chlorophyll a was extracted with 10 ml of 90% acetone overnight at 4 °C in the dark and filtered through GF/F filters to remove any remaining suspended material. Chlorophyll a concentration was determined fluorometrically. Samples that exceeded the range were successively diluted by half with 90% acetone. Chlorophyll concentration was standardized to leaf area (using the previously measured leaf width on that date/treatment × 1 cm leaf length) and leaf weight (using the previously determined area: weight relationship on the epiphyte-free leaves as described above). For the two chlorophyll metrics (concentration and content), the final treatment means for each species/life stage were analyzed by one-way ANOVA followed by Tukey's post-hoc test.

2.7. Chlorophyll Fluorescence

Plants were tested for photosynthetic yield (Fv/Fm) over 3 sequential days at the start of the experiment, again after the first light-deprivation stress period (either 3 or 9 days duration), and at the termination of the trial. Plants were also tested on day 7 of recovery at the end of the trial, the same day on which all remaining plants were collected for destructive sampling of biomass, morphology, and leaf chlorophyll.

Leaves from three randomly selected shoots on different plants in each of the replicate tank partitions were removed for yield measurements so that no leaf was ever measured twice. The mid section of the first mature leaf was chosen, thus avoiding differences in fluorescence and chlorophyll due to factors such as leaf age (Durako and Kunzelman, 2002). The leaf was trimmed at the base of the shoot, placed in the dark, and within <30 minutes of collecting, scraped of epiphytes, blotted dry, placed in a leaf-clip in a darkened, temperature controlled room, and analyzed for Fv/Fm after a 1 second pulse of saturating light from a Plant Efficiency Analyzer or PEA (Hansatech, Kings Lynn, England).

Mortality affected the ability to collect data from all treatments at all time periods, resulting in an unbalanced data set, which prohibited the analysis of the fluorescence data by two-way ANOVA (treatment × time). For this reason, the data were analyzed for selected time points with separate one-way ANOVA. The one-way ANOVAs were performed on the treatment means for the initial (day 0), day 1 post stress at the beginning and end of the experiment, as well as on day 7 post-stress at the termination of the trial. These measurement times represented the greatest stress response or maximum recovery from stress for the entire experiment. Each species and/or life-stage was analyzed separately, with the alpha level of significance adjusted by the Dunn-Sidak method (Sokal and Rohlf, 1995) to correct for non-independence of time.

3. Results

3.1. Abiotic variables

Temperature and salinity were lower in the spring during the *Z. marina* experiment, than in the summer during the *H. wrightii* experiment (Table 1A). Turbidity was low (≤10 NTU) in this semi flow-through system as a consequence of the particle settling tank and filtration unit. There was minimal light attenuation in the water column, with average PAR at the canopy measuring 56% surface irradiance (SI) during the *Z. marina* experiment and 54% SI in the *H. wrightii* trial. Light levels available at the canopy to the seagrass plants ranged from 4.4 - 20.6 mol m^{-2} day^{-1} in the *Z. marina* experiment, and from 1.4 - 21.6 mol m^{-2} day^{-1} in the *H. wrightii* experiment; the two species experienced similar average daily irradiances in the 6 treatments despite seasonal changes in solar irradiance (Table 1B).

Table 3
Biomass and leaf chlorophyll (mean ± S.D.) of two seagrasses, *Zostera marina* in mature and seedling life stages and *Halodule wrightii* in mature life stage only

Life Stage Treatment	Biomass Above (mg)	Biomass Below (mg)	chl a (μg mg^{-1})	chl a (μg cm^{-2})
Zostera – Mature				
Initial	nd	nd	10.15 ± 2.56	13.01 ± 4.52
Light	135.2 ± 21.45 ^{A}	155.7 ± 15.92 ^{A}	17.00 ± 4.14 ^{A}	19.76 ± 4.73 ^{A}
3D:9L	115.2 ± 25.23 ^{A}	96.5 ± 15.08 ^{AB}	9.50 ± 5.60 ^{B}	11.98 ± 6.66 ^{B}
3D:3L	41.4 ± 6.53 ^{B}	76.7 ± 9.34 ^{BCD}	11.06 ± 2.51 ^{B}	17.07 ± 4.06 ^{AB}
9D:9L	29.9 ± 4.59 ^{B}	47.9 ± 6.27 ^{CD}	10.52 ± 2.79 ^{B}	14.86 ± 4.03 ^{AB}
9D:3L	30.2 ± 5.16 ^{B}	95.0 ± 14.93 ^{ABC}	7.09 ± 0.51 ^{B}	11.04 ± 1.01 ^{B}
Dark	8.9 ± 1.79 ^{C}	46.8 ± 8.17 ^{D}	7.08 ± 1.82 ^{B}	9.91 ± 2.44 ^{B}
Zostera - Seedling				
Initial	nd	nd	7.84 ± 1.51	13.92 ± 3.85
Light	40.8 ± 6.06 ^{AB}	39.8 ± 7.07 ^{A}	14.82 ± 7.82 ^{A}	18.23 ± 8.72 ^{A}
3D:9L	53.2 ± 9.90 ^{A}	45.9 ± 8.65 ^{A}	12.24 ± 2.45 ^{AB}	16.51 ± 3.17 ^{A}
3D:3L	19.3 ± 3.63 ^{AB}	28.9 ± 7.40 ^{A}	8.45 ± 0.94 ^{ABC}	14.11 ± 1.64 ^{A}
9D:9L	16.5 ± 2.89 ^{B}	29.8 ± 7.02 ^{A}	10.93 ± 3.92 ^{AB}	14.93 ± 5.69 ^{A}
9D:3L	4.0 ± 1.54 ^{C}	9.5 ± 3.86 ^{B}	7.46 ± 1.31 ^{BC}	10.23 ± 2.30 ^{AB}
Dark	0.4 ± 0.44 ^{C}	3.8 ± 1.77 ^{B}	4.14 ± 2.60 ^{C}	5.51 ± 3.45 ^{B}
Halodule – Mature				
Initial	nd	nd	11.11 ± 0.47	23.15 ± 0.99
Light	2.0 ± 0.67 ^{B}	24.3 ± 5.19 ^{B}	7.98 ± 0.81 ^{A}	16.62 ± 1.67 ^{A}
3D:9L	5.9 ± 1.34 ^{A}	37.5 ± 9.81 ^{B}	6.74 ± 0.53 ^{A}	14.05 ± 1.11 ^{A}
3D:3L	0.5 ± 0.40 ^{B}	22.0 ± 10.27 ^{B}	6.32 ± 1.93 ^{A}	13.17 ± 4.03 ^{A}
9D:9L	2.3 ± 0.20 ^{B}	33.5 ± 9.60 ^{B}	6.68 ± 1.31 ^{A}	13.91 ± 2.74 ^{A}
9D:3L	0.1 ± 0.00 ^{B}	43.4 ± 0.00 ^{A}	1.67 ± 0.14 ^{B}	3.48 ± 0.30 ^{B}
Dark	0.0 ± 0.00 ^{B}	0.0 ± 0.00 ^{B}	0.0 ± 0.00 ^{B}	0.0 ± 0.00 ^{B}

Sample size per treatment was n = 10 for biomass and n = 6 for chl a measurements. Superscripts are means that are not significantly different from one another by Tukey's HSD post-hoc comparison. "nd" indicates no data was collected for that sample.

3.2. Survival

Mortality of plants increased as light-deprivation stress increased for both species and life stages. Mortality was 100% for all plants of both species in the dark treatment (Fig. 1). For both mature and seedling stages of *Z. marina*, mortality was <10% in the light control (L) and the 3D:3L treatment. In contrast to the low mortalities seen in the *Z. marina* experiment, there was substantially higher mortality of *H. wrightii* across all 6 treatments (Fig. 1).

3.3. Morphology

The proportion of the population with 2-3 shoots was very similar at the beginning of the experiment, 78.7% in mature *Z. marina* plants (Fig. 2A), 84.7% in seedlings (Fig. 2B), and 82.8% in *H. wrightii* (Fig. 2C). At the end of the experimental period, there was decline in the number of shoots in both life-stages of *Z. marina* (Fig. 2A, B). In *H. wrightii* there was a decline in the number of shoots from 2 to 1 in surviving plants (Fig. 2C).

The average number of leaves per shoots decreased during the experiment in all *Z. marina* plants (Table 2). Number of leaves per shoots decreased significantly in both mature *Z. marina* plants ($F_{5,215}=12.6899$, $P<0.0001$) and seedlings ($F_{5,215}=30.0345$, $P<0.0001$). There was no significant treatment effect on number of leaves per shoots in *H. wrightii* other than D ($F_{5,101}=10.6981$, $P<0.0001$).

Average leaf length increased throughout the experiment in the L and 3D:9L treatments in both life stages of *Z. marina* plants (Table 2). Leaf length declined significantly as stress increased in both the mature ($F_{5,215}=39.1813$, $P<0.0001$) and seedling ($F_{5,215}=42.5668$, $P<0.0001$) stages of *Z. marina*. In contrast, most plants of *H. wrightii* had significantly shorter ($F_{5,101}=15.2394$, $P<0.0001$) mean leaf lengths at the end of the experiment (Table 2).

In the mature plants there were no significant differences in average leaf width by treatment, except for D (*Z. marina* $F_{5,215}=6.8819$, $P<0.0001$, and *H. wrightii* $F_{5,47}=4.6129$, $P=0.0019$, Table 2). In *Z. marina*

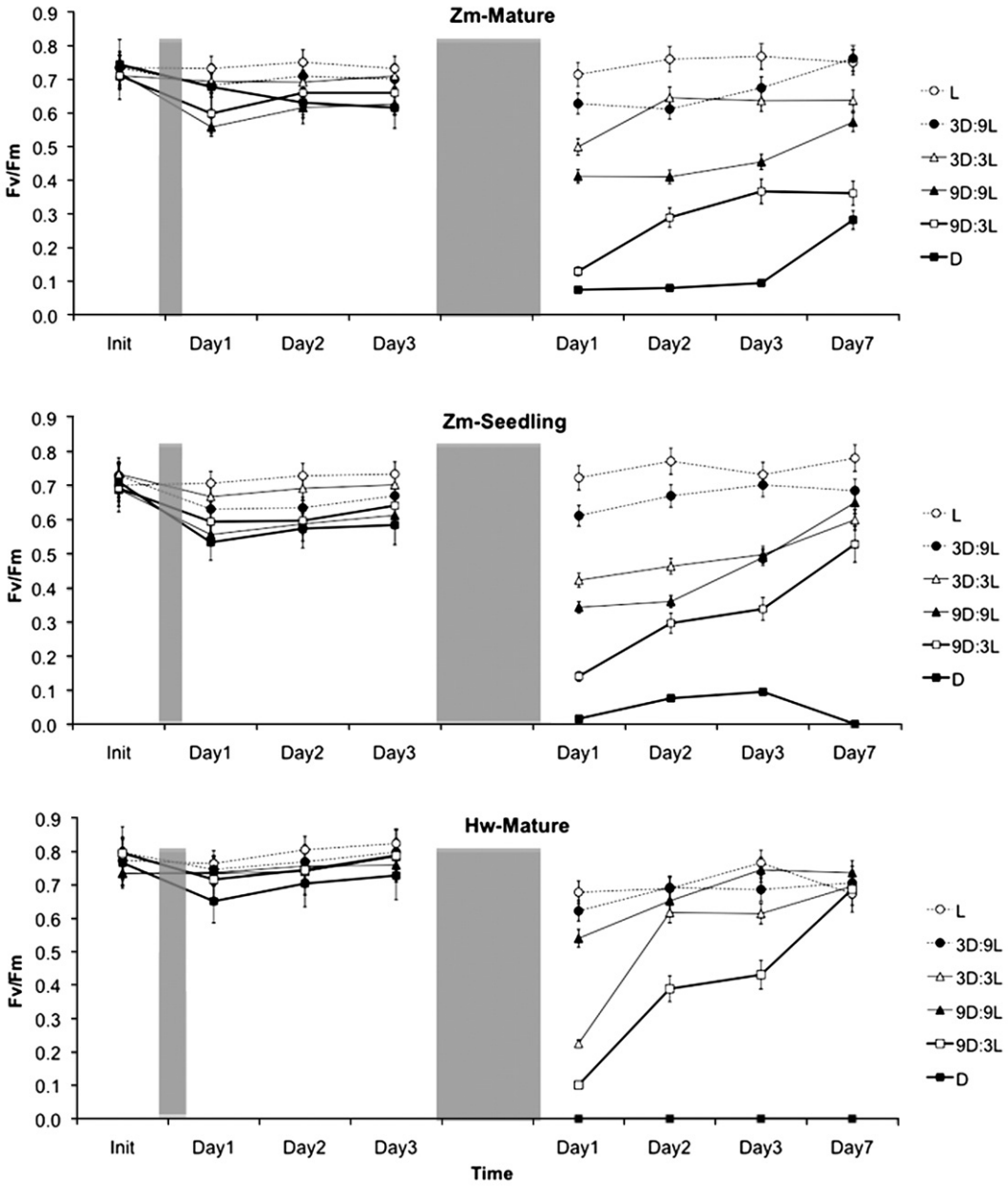

Fig. 3. Chlorophyll fluorescence (Fv/Fm) response of *Zostera marina* (Zm) seedlings and mature plants and *Halodule wrightii* (Hw) measured over 3 consecutive days to assess recovery after light-deprivation stress events that were 3 days or 9 days in duration. Grey horizontal bars indicate single or multiple stress events.

Table 4
Results of one-way ANOVA comparisons of mean Fv/Fm by treatment on selected days of the *Zostera marina* and *Halodule wrightii* experiments presented in Fig. 3

Species/Stage	Day 0 - initial	Day 1 first stress event	Day 1 last stress event	Day 7 – termination
Zostera - Mature	$F_{5,66}=1.3080$	$F_{5,66}=16.7031$	$F_{5,60}=9.7891$	$F_{5,28}=7.2495$
	$P_4=0.2714$	$P_1<0.0001$	$P_2<0.0001$	$P_3=0.0002$
Zostera - Seedling	$F_{5,66}=3.7072$	$F_{5,66}=16.0631$	$F_{4,55}=12.3868$	$F_{4,24}=2.0293$
	$P_3=0.0051$	$P_1<0.0001$	$P_2<0.0001$	$P_4=0.1253$
Halodule - Mature	$F_{5,66}=2.7345$	$F_{5,27}=7.1631$	$F_{4,25}=51.0875$	$F_{4,31}=2.5225$
	$P_3=0.0263$	$P_2=0.0002$	$P_1<0.0001$	$P_4=0.0609$
Dunn-Sidak adjusted alpha	$P_1\leq0.0127$	$P_2\leq0.0169$	$P_3\leq0.0253$	$P_4\leq0.05$

Sample size per treatment was n=6 on each day. Alpha levels of the ranked P values were Dunn-Sidak adjusted to compensate for non-independence of time. The appropriate adjusted alpha for each ANOVA test is indicated by subscript on the P value and should be compared with the critical values calculated by the Dunn-Sidak test at the bottom of the table.

seedlings there was a significant decline in width ($F_{5,215}=44.0650$, $P<0.0001$) with increasing stress (Table 2).

3.4. Biomass

Above-ground shoot and leaf biomass declined significantly as stress increased (Table 3). There were significant differences among treatment means in the mature *Z. marina* ($F_{5,59}=26.9419$, $P<0.0001$) and seedlings ($F_{5,59}=38.7121$, $P<0.0001$), with highest above-ground biomass found in L and 3D:9L. The only treatment that was significantly different ($F_{5,59}=19.7908$, $P<0.0001$) in the *H. wrightii* experiment was 3D:9L; this was the only treatment with more than 10 plants remaining at the end of the experiment.

Below-ground root and rhizome biomass followed the pattern established in the above-ground data. Significant differences occurred among treatment means, with higher below-ground biomass in the less stressed mature ($F_{5,59}=9.2987$, $P<0.0001$) *Z. marina* and seedlings ($F_{5,59}=13.5911$, $P<0.0001$). The only treatment that was significantly different ($F_{5,59}=9.8781$, $P<0.0001$) in the *H. wrightii* experiment was 9D:3L.

3.5. Leaf Chlorophyll

Chlorophyll a (chl) concentration ($\mu g\ mg^{-1}$) and content ($\mu g\ cm^{-2}$), were higher in the less stressed plants than those in the more light-deprived treatments (Table 3). At the start of the experiments, chl concentration was similar in the mature plants of both species, but lower in *Z. marina* seedlings (Table 3). (Table 3). There was an increase in the chl concentration and content in the light treatment over the 40 day experiment in *Z. marina*; this was not the case in *H. wrightii* (Table 3).

For both life-stages of *Z. marina* there was a significant decline in chl concentration (mature $F_{5,35}=7.8972$, $P<0.0001$; seedling $F_{5,35}=6.2573$, $P<0.0004$) and content (mature $F_{5,35}=5.0688$, $P=0.0017$; seedling $F_{5,35}=5.7123$, $P=0.0008$) as light-deprivation stress increased. Significant reductions were also evident in the leaf chl *a* concentration ($F_{5,35}=9.8322$, $P<0.0001$) and content ($F_{5,35}=9.8314$, $P<0.0001$) for *H. wrightii* (Table 3).

3.6. Chlorophyll Fluorescence

Both seedling and mature *Zostera marina* and mature *H. wrightii* plants, exhibited similar reductions in dark adapted photosynthetic yield (Fv/Fm) due to light-limitation stress (Fig. 3). As stress duration increased (9 vs. 3 days), there was a corresponding decrease in Fv/Fm, with recovery to pre-stress levels taking longer as the duration of the stress became longer. Recovery time (3 vs. 9 days) also affected the recovery of photosynthetic efficiency. Plants with long stress and short recovery (9D:3L) periods were least able to return to the pre-stress Fv/Fm levels (Fig. 3).

There was no significant difference in Fv/Fm in the L treatment over time for mature plants ($F_{7,89}=2.0551$, $P=0.1011$) and seedlings ($F_{7,89}=2.2175$, $P=0.0807$) of *Z. marina*. The Fv/Fm ratio in the L treatment changed significantly ($F_{7,53}=3.7890$, $P=0.0025$) in *H. wrightii* during the course of the experiment, declining over time (Fig. 3).

After the first shading event, *Z. marina* plants that had been deprived of light for 9 days had significantly lower Fv/Fm than those that were subject to only 3 days shading (Table 4). Similar patterns were evident in *H. wrightii* plants, even though Fv/Fm did not drop as much compared to *Z. marina* (Fig. 3).

After multiple shading events, plants that were exposed to stress exceeding recovery time (9D:3L and D) had experienced substantial mortality in both species and life-stages, and this was evident in the low Fv/Fm (Fig. 3). Both life stages of *Z. marina* and mature *H. wrightii* in the L and 3D:9L treatments had significantly greater Fv/Fm immediately after the last stress event than the other four treatments (Table 4). After one week recovery, all plants in the L, 3D:9L, and 3D:3L treatments measured Fv/Fm no less than that after the first shading event (Fig. 3).

There were no significant differences among the treatments on day 7 of recovery for *Z. marina* seedlings and *H. wrightii* (Table 4), while for mature *Z. marina*, some lower Fv/Fm ratios occurred in 9D:3L and D (Fig. 3). Recovery of Fv/Fm in D plants over the 7 day recovery period at the end of the experiment was not observed (Fig. 3).

Both *H. wrightii* and the two life-stages of *Z. marina* exhibited similar stress-recovery patterns in photosynthetic yield. After the first light-deprivation period (3 or 9 days) plants were generally able to recover to pre-stress performance levels within 3 days. However, after repeated light-deprivation events, plants in the treatments where stress: recovery time ≥1:1 exhibited significant decreases in Fv/Fm ratios.

4. Discussion

4.1. General treatment responses

Lengthy, repeated periods of light-deprivation caused higher mortality of shoots and individuals in these two seagrass species. Individuals of both species and of specific life-stages produced fewer or no new shoots as light-deprivation periods increased in duration and frequency. The light control (L) and low stress (3D:9L) did better than the 1:1 stress: recovery treatments (3D:3L and 9D:9L), which did better than stress exceeding recovery (9D:3L) and the dark control (D). In the latter part of the experiment, we observed that many plants in the dark control appeared to still have healthy leaves (green pigment and fluorescence), however, even gentle pulling caused the leaves to separate from the rhizome indicating breakdown of shoot tissue. These results suggest that a recovery period should equal to or exceed the duration of light-deprivation stress period for long-term plant survival.

Morphology and biomass integrate plant responses over days to weeks and are frequently used by seagrass researchers to assess plant responses to environmental conditions (Short and Duarte, 2001). Above-ground biomass, number of leaves per shoot, and leaf length were all found to be superior at capturing shading stress than either leaf width, number of shoots per plant, or below-ground biomass during these experiments. As stress increased, new leaf production was either severely reduced or resulted in slower production of new leaves that were thinner and shorter. Mortality of the basal meristem occurred in many plants in the more stressful treatments and caused the death of the plant and loss of all above-ground tissues. Below-ground tissue in the dark control were observed to be less viable than the other treatments, with few portions floating in water after rinsing off the sediments (an indication that lacunae were no longer replete

with oxygen), suggesting that below-ground tissues were probably dead (Longstaff et al., 1999).

Recent investigations of stress responses in seagrasses, particularly under chronic light limitation, have focused on photosynthetic rates and concentrations of pigments as early indicators of chronic stress (Burke et al., 1996; Longstaff et al., 1999). Reductions in chlorophyll pigment content, and changes in Fv/Fm, due to leaf senescence were observed in both species and life-stages in our experiments. The chlorophyll responses measured are interesting, as seagrasses in low light environments have been found to produce more chlorophyll, presumably to better intercept available light (Major and Dunton, 2002; Cummings and Zimmerman, 2003). However, if light deprivation continues, then pigments and other carbon reserves begin to break down as the plant attempts to maintain enough energy to cover respiratory demands (Zimmerman et al., 1995; Zimmerman and Alberte, 1996). Reduction in pigment content creates a disadvantage in terms of the plant's ability to harvest a low flux of photons resulting in the need to consume further structural carbon. This quickly leads to tissue senescence and mortality from a lack of energy to support cellular metabolism (Touchette and Burkholder, 2000).

The chlorophyll fluorescence results mirrored closely the morphological and physiological changes described above. The chlorophyll fluorescence technique generates a yield efficiency ratio, Fv/Fm, which has been interpreted as a measure of stress (Genty et al., 1989). The greater the fluorescence the higher the efficiency of photosystem II, which equates with a leaf sample under low physiological stress; conversely, low Fv/Fm is often interpreted to indicate high stress (Maxwell and Johnson, 2000). In our experiment, chlorophyll fluorescence was a sensitive measure of plant stress. In all but the light control, plants indicated a slight to substantial decline in Fv/Fm immediately post stress, and the recovery phase lasted for 3 to >7 days after removal of the shade-stress. Not surprisingly, plants with short stress periods and longer intervals between stress (e.g., 3D:9L) had higher photosynthetic yield than those with longer stress and shorter recovery periods.

4.2. Life-stage and species differences

Zostera marina plants were selected to investigate the potential difference in stress-tolerance due to life-stage. Loss of individuals at the juvenile stage is especially critical for population maintenance and future growth. Furthermore, *Z. marina* seedlings are often observed to grow at the edges of a bed, because mature plants within the bed already occupy all the space and/or consume available nutrient resources (Olesen and Sand-Jensen, 1994; Olesen et al., 2004). Growing at the deep-edge of the meadow results in high susceptibility to light-limitation, and hence high potential mortality. However, *Z. marina* seedlings did not appear to show greater effects of light-deprivation stress than the mature life-stage. Lower root and shoot biomass may be an advantage to seedlings because stress could be related to oxygenating the rhizosphere. Seedlings also have less above-ground tissue, so stored carbohydrate resource requirements to meet metabolism are lower as there is less tissue to maintain. These results suggest that seedlings are no less tolerant of deep-edge conditions than the mature plants and may not be at a strong disadvantage, as they require fewer resources because of their smaller mass (Bintz and Nixon, 2001).

Different responses were observed between the mature plants of the two species. The lack of survival/growth in *H. wrightii* may have been that the two shoot experimental planting unit was too small. For instance *H. wrightii* fragments with 3 shoots were only able to survive 2-4 weeks (Hall et al., 2006), and for restoration transplanting it is recommended plants have 5-15 shoots (Fonseca et al., 1998).

A further cause of the higher morality in the *H. wrightii* experiment may be related to it's approximately 50% higher light requirements than *Z. marina* (Dunton, 1994; Dunton and Tomasko, 1994; Dennison and Alberte, 1985; Mazella and Alberte, 1986). *Zostera marina* plants received sufficient light to saturate growth ($H_{sat} \geq 9$) in all treatments with the exception of the 9D: 3L stress and D control (Table 1B). In contrast, *H. wrightii* with its' higher light requirements was potentially light limited ($H_{sat} < 8$) in all treatments but the L control.

4.3. Fluorescence and monitoring

The results from this study show strong coupling of Fv/Fm and plant morphometrics, biomass, and pigment content, after acute shading stress. Fv/Fm was a responsive measure of condition and recovery, especially in the intermediate stress levels. This agrees with previous studies that have demonstrated that Fv/Fm metrics are appropriate for looking at acute stress recovery dynamics (Ralph and Burchett, 1995; Ralph, 1998) more so than at chronic adaptable stress events (Silva and Santos, 2003; Strain et al., 2006). However, fluorescence measurements only incorporate portions of a leaf and may become difficult to extrapolate to population levels due to high levels of uncertainty (Durako and Kunzelman, 2002; Cayabyab and Enriquez, 2007).

Chlorophyll fluorescence responses should be integrated with other measures of plant condition, such as morphology, biomass, and/or cellular constituents for Fv/Fm to be put in the appropriate long-term context and make inferences about the overall condition of the plant. The problem may arise, as seen in this study, of plant mortality occurring at the meristem while the existing photosynthetic units (leaves) were observed to continue to exhibit very satisfactory Fv/Fm measurements. For these reasons, it is unlikely that chlorophyll fluorescence can be used as the sole metric to determine seagrass condition and should be approached with caution when no other data is available to place the Fv/Fm measurements in an appropriate context.

Only seagrasses in treatments where light-deprivation was followed by a recovery interval of at least the same duration (3 or 9 days) showed signs of long-term survival, irrespective of species or life-stage. These results suggest that seagrasses occurring at the deep-edges are strongly susceptible to light-deprivation events, and need recovery times of at least the duration of the previous attenuation event. For instance, Longstaff et al. (1999) reported little recovery of seagrass was observed after a 40 day attenuation event. Because light is already a limiting resource at the deep-edge, these plants are frequently and disproportionately affected by attenuation events that may less severely affect the rest of the meadow. Future work in this area should focus on determining integrated plant and population survival models for predictive forecasting of the consequences of light attenuation events that may be of concern for seagrass resources.

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