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Tropical seagrass species tolerance to hypersalinity stress

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Abstract

The long-term sustainability of seagrasses in the subtropics and tropics depends on their ability to adapt to shifts in salinity regimes, particularly in light of present increases in coastal freshwater extractions and future climate change scenarios. Although there are major concerns world-wide on increased salinity in coastal estuaries, there is little quantitative information on the specific upper salinity tolerance of tropical and subtropical seagrass species. We examined seagrass hypersalinity tolerance under two scenarios: (1) when salinity is raised rapidly simulating a pulsed event, such as exposure to brine effluent, and (2) when salinity is raised slowly, characteristic of field conditions in shallow evaporative basins; the first in hydroponics (Experiments I and II) and the second in large mesocosms using intact sediment cores from the field (Experiment III). The three tropical seagrass species investigated in this study were highly tolerant of hypersaline conditions with a slow rate of salinity increase (1 psu d^{-1}). None of the three species elicited total shoot mortality across the range of salinities examined (35–70 psu over 30 days exposures); representing in situ exposure ranges in Florida Bay, a shallow semi-enclosed subtropical lagoon with restricted circulation. Based on stress indicators, shoot decline, growth rates, and PAM florescence, all three species were able to tolerate salinities up to 55 psu, with *Thalassia testudinum* (60 psu) and *Halodule wrightii* (65 psu) eliciting a slightly higher salinity threshold than *Ruppia maritima* (55 psu). However, when salinity was pulsed, without a slow osmotic adjustment period, threshold levels dropped 20 psu to approximately 45 psu for *T. testudinum*. While we found these three seagrass species to be highly tolerant of high salinity, and conclude that hypersalinity probably does not solely cause seagrass dieoff events in Florida Bay, high salinity can modify carbon and O_2 balance in the plant, potentially affecting the long-term health of the seagrass community.

Keywords: Salinity; Hypersalinity; Seagrass; *Thalassia testudinum* Banks ex Konig; *Halodule wrightii* Aschers.; *Ruppia maritima*; Osmotic adjustment

1. Introduction

Seagrasses are keystone species in many shallow lagoons and estuaries providing a complex habitat and high rates of primary production for ecologically and economically important higher consumers (Bell and Pollard, 1989; Heck et al., 2003; Bloomfield and Gillanders, 2005). The long-term sustainability of seagrasses, particularly in the subtropics and tropics, depends on their ability to adapt to shifts in salinity regimes influenced by anthropogenic modifications of upstream hydrology, as well as long-term temperature increases predicted to be associated with future climate change (Short and Neckles, 1999). Seagrass species in the subtropics may be more susceptible to moderate increases in heat loads because they already exist at their upper physiological tolerance to temperature and salinity, although few studies have confirmed upper thresholds. Susceptibility to these stressors is most pronounced in shallow lagoons with restricted circulation. For example, Florida Bay (1800 km^{-2}), a shallow semi-enclosed lagoon in South Florida, reaches salinities greater than 35 psu almost every year in the recent past (1989–2002; Fig. 1), and has been found to sustain hypersaline conditions year-round during periods of drought (Fig. 1; Boyer et al., 1999; Fourqurean and Robblee, 1999). In addition to natural periodicities of drought in the subtropics, coastal freshwater extractions reduce freshwater inflows to estuarine systems, important for balancing high rates of evaporation. For example, the diversion and withdrawal of freshwater from the Caloosahatchee River on the west coast of Florida has resulted in negative ecological consequences for the Caloosahatchee Estuary, such as highly variable salinity and loss of submerged aquatic vegetation (Doering et al., 2002). In Florida Bay, hydrological modifications in the highly engineered Greater Everglades system significantly influence the amount and

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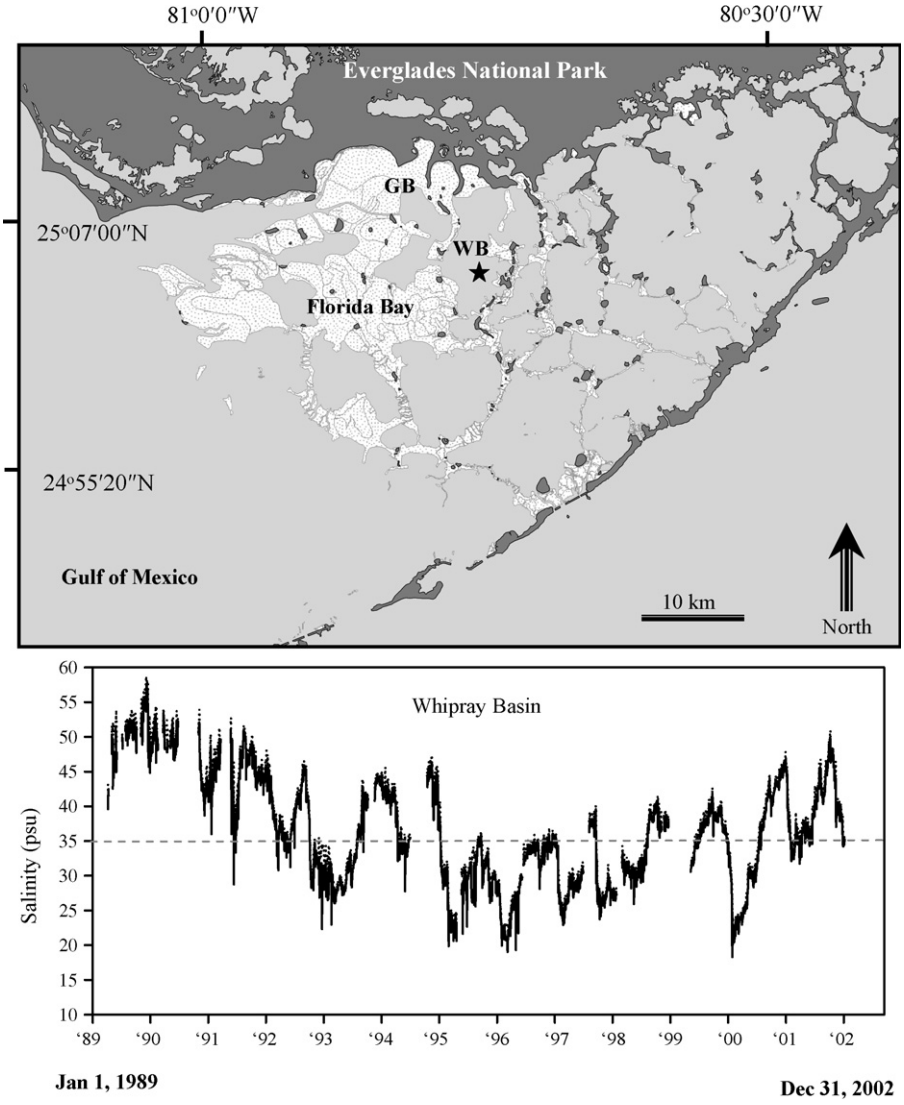

Fig. 1. Seagrass collection sites in Whipray Basin (WB) and Garfield Bight (GB) in north central Florida Bay at the lower terminus of the Florida peninsula south of the freshwater everglades (upper panel). Long-term daily minimum and maximum salinity data for WB from 1989 to 2002 (lower panel).

timing of freshwater flows received by the Bay (Light and Dineen, 1994; Nuttle et al., 2000). Upstream hydrologic changes, combined with the seasonality of rainfall, define the salinity envelope of these and other systems and determine seagrass exposure to hypersaline conditions.

Although there are major concerns world-wide on the potential and in some cases present increases in salinity of coastal estuaries (Short and Neckles, 1999), there is very little quantitative information on the specific upper salinity tolerances of tropical and subtropical seagrass species. Salinity in natural estuarine and lagoon systems increase at slow rates due to the synergistic effects of evaporation and restricted circulation, such as in Florida Bay (Nuttle et al., 2000), Shark Bay, Western Australia (Walker et al., 1988), and Baffin Bay, Texas (Cotner et al., 2004). However, desalination plants that use reverse osmosis for fresh water supply, such as along the Mediterranean and elsewhere in the world, have resulted in hypersaline effluent (44–90 psu) being pulsed into coastal systems (Fernandez-Torquemada and Sanchez-Lizaso, 2005). Thus, it is important to evaluate seagrass salinity tolerance to both pulsed and a slow rates of salinity increase.

One of the limitations in defining specific upper salinity tolerances in coastal marine environments is the difficulty of conducting controlled field experiments, particularly in subtropical and tropical environments where rainfall is spatially variable and salinities are highly dynamic. In addition, interactive stressors such as high temperature can confound the determination of a hypersalinity response in the field where heat loads drive evaporation and hypersaline conditions. Thus, we used a highly controlled mesocosm experimental approach to articulate the upper salinity tolerances of three dominant tropical seagrass species, *Thalassia testudinum* Banks ex König, *Halodule wrightii* Aschers. and *Ruppia maritima* L. Although we recognize that *R. maritima* is not a true seagrass, it is a dominant submerged macrophyte found at the freshwater Everglades-Florida Bay marine ecotone, a zone that becomes hypersaline during droughts. This seagrass species is also predicted to increase in distribution in Florida Bay with greater

freshwater flows to the Bay resulting from Everglades Restoration (Fourqurean et al., 2003) and thus its salinity tolerance needs to be further evaluated.

We examined seagrass hypersalinity tolerance under two scenarios: (1) when salinity is raised rapidly simulating a pulsed exposure event and (2) when salinity is raised slowly characteristic of field conditions in shallow evaporative basins. Both scenarios were examined for *T. testudinum*, the dominant bed forming seagrass in Florida Bay and a dominant species in the wider Atlantic–Caribbean region. Upper hypersalinity thresholds were compared amongst three dominant species in the Bay (*T. testudinum*, *H. wrightii* and *R. maritime*) under a slow rate of salinity increase. We hypothesized that slow rates of salinity increase would allow time for plant osmoregulation, and thereby sustain growth of *T. testudinum* across a broader range of hypersaline conditions compared to thresholds established using pulsed experiments. While short-term pulsed experiments were conducted hydroponically, a large mesocosm system (16 500 L tanks) was constructed for longer-term experiments applying a gradual rate of salinity increase to intact seagrass cores from the field.

2. Materials and methods

2.1. Pulsed hypersalinity Experiments (I and II)

2.1.1. Field plant sampling—T. testudinum

Intact *T. testudinum* shoots were carefully removed from the sediment throughout a central basin of Florida Bay that frequently becomes hypersaline (Garfield Bight to Whipray Basin, Fig. 1). Care was taken to extract large sections of rhizomes in order to maintain intact rhizome-connected shoots. Once plants were brought to the boat, they were stored in a large cooler (30 L) with ambient seawater. The cooler was transported to the Florida Atlantic University Marine Lab in Boca Raton, FL. Within 24 h, plants were taken from coolers and placed in a large tank with circulating seawater and a large aquarium pump. Light approximated saturation (~500 μmol photons m^{-2} s^{-1}; Fourqurean and Zieman, 1991) using two 1000 W metal halide lights maintained on a 12 h:12 h light:dark cycle. Plants from this sampling event were kept under saturating light and ambient salinity (35 psu, coastal Atlantic) before being used in the short-term Experiments I and II.

2.1.2. Pulsed hydroponic experiment design

Split compartment chambers (Fig. 2a; 50 cm height × 15 cm diameter; 4 L upper compartment, 2 L lower compartment) were used in two short-term pulsed salinity hydroponic experiments (3 days). In Experiment I, we included the upper range of field salinity recorded in Florida Bay (70 psu; Zieman, pers. commun.) to establish the upper level of salinity tolerance for *T. testudinum* under pulsed treatments. The lowest salinity treatment used was the field salinity measured (45 psu) when plants were collected July 2001. The specific salinity treatment levels that were applied to the upper compartment of the chambers were as follows: 45, 50, 55, 60, 65, and 70 psu and root chambers were held to a constant salinity of 40 psu. Each salinity level was tested in replicate chambers giving a total of ten chambers for Experiment I.

For Experiment II, we tested the lower range of salinity including ambient and up to 55 psu: 35 (control), 40, 45, 50, and 55 psu ($n = 3$ chambers per treatment). Root compartments were amended with ambient seawater (35 psu) to reduce any stress observed in Experiment I by having both leaf and root compartments under hypersaline conditions (>35 psu).

In the hydroponic setup (Fig. 2a) above and belowground tissues were isolated via a central disc. Prior to plant insertion into the chambers, the leaf base was marked with a needle to gauge leaf elongation rates over the course of the experiment (adapted from Zieman, 1974). In four of the holes rhizome-connected shoots were inserted with at least 5–10 cm of rhizome segment attached and the fifth central hole was sealed with a rubber stopper. Each plant was sealed into a stopper with non-toxic sealant (plumber's putty) to eliminate water exchange between the top and bottom of the chamber.

Salinity levels for the two experiments were achieved by amending artificial salts (Instant Ocean Inc.) to ambient coastal seawater (35 psu). Immediately after plants were inserted into chambers, seawater treatments (described above) were added to both bottom and top compartments and chambers were O-ring sealed. Once chambers were filled with seawater, they were carefully placed in a large tank with flowing seawater to maintain temperatures below 30 °C. For pulsed salinity experiments, light levels above the tank were maintained as described above.

2.1.3. Net photosynthesis and leaf elongation measurements

In Experiments I and II, rates of photosynthesis were determined by measuring oxygen (O_2) evolution in the upper chamber over time. Oxygen measurements were initiated 45 min after the beginning of the light cycle in the morning (0800 h). Oxygen (mg L^{-1} and % saturation) was recorded every 15 min with approximately five to six measurements taken over a 3–4 h period. The O_2 sensor was equipped with an electric stirrer and an aquarium pump was placed in each chamber to keep the water well circulated. Excellent linear regression coefficients (>0.90 R^2; O_2 as function of time in min) were obtained over the five to six readings taken each day. Chamber productivity was normalized to total photosynthetic biomass in the chamber and is reported as mg O_2 g DW^{-1} h^{-1}.

Growth rates were determined as leaf extension and new biomass based on the leaves marked before the experiment commenced and after 3d at treatment. High precision digital calipers were used to measure leaf elongation to 0.1 mm. The sections of leaf associated with new growth were cut, dried and weighed. Growth rate is reported as new leaf biomass normalized to total photosynthetic biomass per chamber (mg g DW^{-1} d^{-1}) and average leaf elongation rate (mm d^{-1}) to compare with mesocosm results in Experiment III.

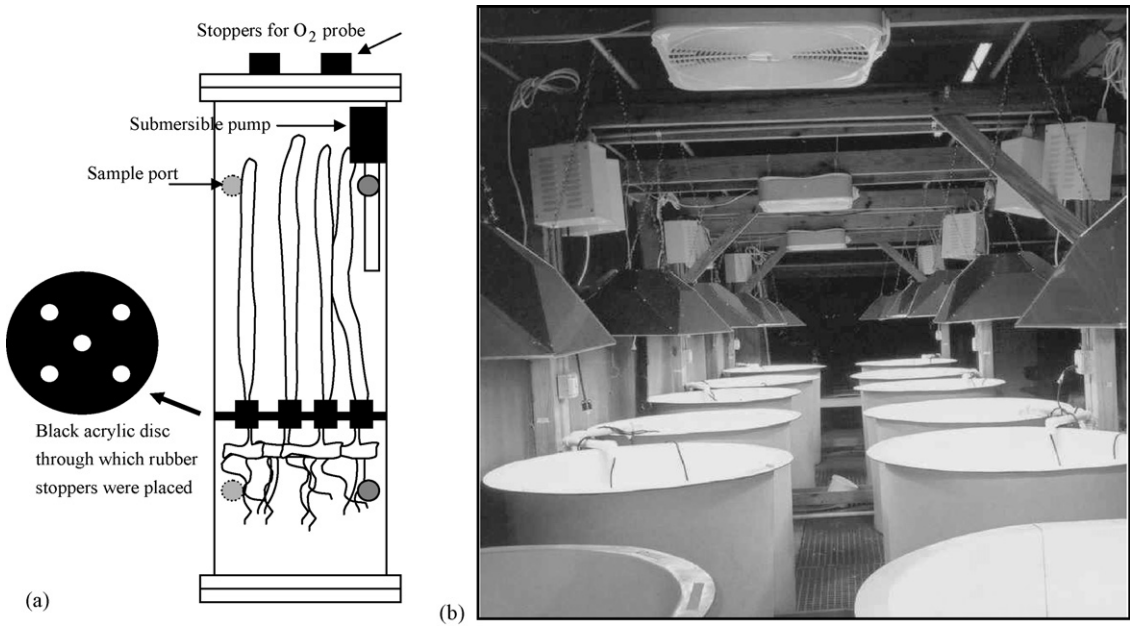

Fig. 2. Hypersalinity experimental setup: (a) split compartment hydroponic growth chambers (Experiments I and II) and (b) large-scale mesocosms (Experiment III).

2.2. Mesocosm osmotic-adjustment Experiment (III)

A large-scale mesocosm setup was constructed for Experiment III (Fig. 2b) which included 16 (500 L; 3 m diameter × 3 m height) fiberglass tanks equipped with two powersweeps, one for circulation at the height of the canopy and the other for surface to bottom circulation and continuous aeration. Each tank had a 1000 W metal halide light with detachable ballast delivering PAR light levels ranging between 1157 and 1304 μmol photons m^{-2} s^{-1} just below the water surface and 726 and 825 μmol photons m^{-2} s^{-1} at the canopy. Light is measured using a spherical quantum sensor (LI-193, LI-COR Inc.) which captures reflected light from the white walls and bottom of the tanks. The temperature-controlled Lab facility maintained the tank water temperature between 27 and 28 °C.

Eight different salinity treatment levels: 35, 40, 45, 50, 55, 60, 65, and 70 psu were examined in replicate tanks with three dominant Florida Bay seagrass species (*T. testudinum* Banks ex König, *H. wrightii* Aschers. and *R. maritima* L.). Intact sediment cores (15 cm diameter × 20 cm depth) of the three seagrass species were collected from north-central Florida Bay (Whipray Basin to Garfield Bight; Fig. 1), transported in coolers, and immediately placed into mesocosms. In addition to intact sediment cores, a second set of experimental units was collected: shoot segments with at least one apical rhizome shoot and four to five live short shoots for each species. At least two shoot-rhizome segments for each species were transplanted into each container (30 cm × 10 cm) with sediment from the field where plants were collected. These two experimental units (intact cores and apical rhizome shoots) were utilized to account for any differences in seagrass stress response due to cut rhizomes of intact cores, a situation more likely problematic in cores of *T. testudinum* because of its larger size than the other two species. In summary, each of the 16 tanks contained 3 intact cores and 3 containers with apical rhizome shoots of *T. testudinum*, *H. wrightii*, and *R. maritima*.

To simulate the in situ rates of salinity increase in the north-central Bay, salinity levels were adjusted using a slow rate of increase. The in situ rates of salinity increase from evaporation, based on long-term data from basins in the Bay, is approximately 0.1–0.2 psu d^{-1} (Fig. 1); however, on the shallow carbonate banks in the Bay (Fig. 1) salinity increases can be on the order of 0.5 psu d^{-1} (Koch unpublished data). We decided on a 1.0 psu d^{-1} in order to get the tanks to treatment salinity in a reasonable amount of time (1 month) and within the growing season, although the rate was faster than long-term data indicate. In addition to increasing salinities slightly faster than in the field, below-ground tissues were exposed to tank treatment salinities, as porewater salinity in the mesocosm cores exchanges more readily than under field conditions (Koch, unpublished data). Therefore, our results are a conservative estimate of stress response to the rates of hypersalinity increases commonly found in the Bay. The experiment was run as a closed system with deionized water amended as needed to compensate for evaporation. Coastal seawater from the flow-through system on each tank was added weekly to maintain nutrient levels in the tanks. C:N (16.3 ± 0.4) and C:P (843 ± 36) molar ratios of *T. testudinum* leaf tissue at the end of the experiment confirm that the plants were not highly deficient in N or P based on nutrient tissue data for this species in general (Duarte, 1990) and more specifically from Florida Bay (Fourqurean et al., 1992). The forgoing stoichiometric nutrient ratios include averages from 35 to 60 psu treatments that were not significantly different from each other; the C:N and C:P ratios from the 65 psu treatment were slightly higher (19 and 1294, respectively) and probably reflect a treatment response. Tank salinity and temperature were

monitored daily, while light and oxygen were measured weekly. The experiment was at treatment salinity levels 21 October 2002 and run for 1 month ending 19 November 2002. During the experiment, ecophysiological and growth response variables were examined for all three species.

2.3. Biological response variables (Experiment III)

T. testudinum growth was estimated by leaf elongation rate (described above), while *H. wrightii* and *R. maritima* new shoot production rates or plastochron interval was used as an indicator of growth, as the very narrow leaf blades of these species do not accommodate a leaf marking technique. Plastochron intervals were determined by tagging apical shoots with small cable ties before being inserted into the sediment at the initiation of the experiment and new shoots counted at the termination of the experiment. In addition to growth estimates, density of shoots was followed by counting live shoots weekly in both cores and tubs for all three species, and is reported as percent change from the initial density. Quantum efficiency of photosystem II or chlorophyll fluorescence (F_v/F_m) was determined on dark adapted (5 min) leaves each week using a Diving PAM (Pulse Amplitude Modulation; Walz, Germany). The F_v/F_m ratio has been applied as an indicator of seagrass stress, with an optimal range for seagrass approximately 0.7–0.8 (Ralph, 1999; Beer et al., 2001; Durako and Kunzelman, 2002), slightly lower than C_3 terrestrial plants (0.83; Björkman and Demmig, 1987). In the presentation of our PAM results, we use a F_v/F_m ratio of 0.7 as a lower threshold.

Once plants were harvested, they were separated into leaves, roots, and rhizomes and immediately frozen in liquid nitrogen and freeze-dried. Total soluble carbohydrates and starch were measured in leaves and rhizomes of all species. Briefly, soluble carbohydrates were extracted from ground tissue with 80% (v/v) aqueous ethanol using methods described in Erskine and Koch (2000) modified from Yemm and Willis (1954). The insoluble carbohydrates (starch) were extracted from the same tissue sample with 3 mL of 0.1 M NaOH overnight at 24 °C (Zimmerman et al., 1989). A sub-sample of leaf tissue was frozen separately for determination of total osmolality using a vapor pressure osmometer (Westcor Inc., Logan, UT, Model 5520). Both *T. testudinum* and *R. maritima* leaves were frozen prior to osmolality readings, giving total osmolality. However, *H. wrightii* leaves had to be measured fresh for stable readings, therefore are assumed to be primarily cytoplasmic non-vacuolar osmolality. Leaf discs of approximately 6.5 mm in diameter were made using a standard hole-punch in triplicate for *T. testudinum* and leaf sections were cut for *H. wrightii* and *R. maritima* to fill the 6.5 mm osmometer chamber. After freezing for 24 h at −4 °C, leaf discs and sections were transferred directly to the osmometer. Prior to readings, the osmometer was sequentially calibrated with 100, 290 and 1000 mmol kg^{-1} standards according to standard methods (Westcor Inc.). To test the time required for equilibration, osmolality readings were gathered in the time delay mode at intervals of 5 up to 40 min; we found osmolality readings stabilized after 20 min.

3. Results

3.1. Pulsed hypersalinity Experiments (I and II)

After pulsed hypersaline treatments, *T. testudinum* showed a positive net production rate in both Experiments I and II after 3 days. However, the rates of photosynthesis were lower as salinity increased, particularly above 45 psu (Fig. 3a). High variance and relatively low power precluded statistically determining an intermediate threshold based on O_2 production, but a difference was found between 35 and 60, 65, and 70 psu when both Experimental (I and II) data sets were pooled (overall significance $P < 0.01$) and applying a Dunnett's test (using 35 psu as control). Leaf growth rates representing net production were more sensitive to hypersalinity treatments (Fig. 3b and c). Leaf growth rates (mm d^{-1}) were significantly different among salinity treatments ($P < 0.01$; Kruskal–Wallis ANOVA) and on average showed a steep decline beyond 45 psu: 39% from 45 and 50 psu and then 42% from 50 to 55 psu (Fig. 3c); however, significant differences were only found between 35 and 55 psu applying a Tukey post hoc test and Bonferroni with 35 psu as a control. While the statistical differences were difficult to define because of high variance, leaf growth rates in salinity treatments ≥50 psu fell below 2.0 mm d^{-1} (Fig. 3c), a critical leaf elongation rate that we have found in our experimental work to indicate stress in *T. testudinum*.

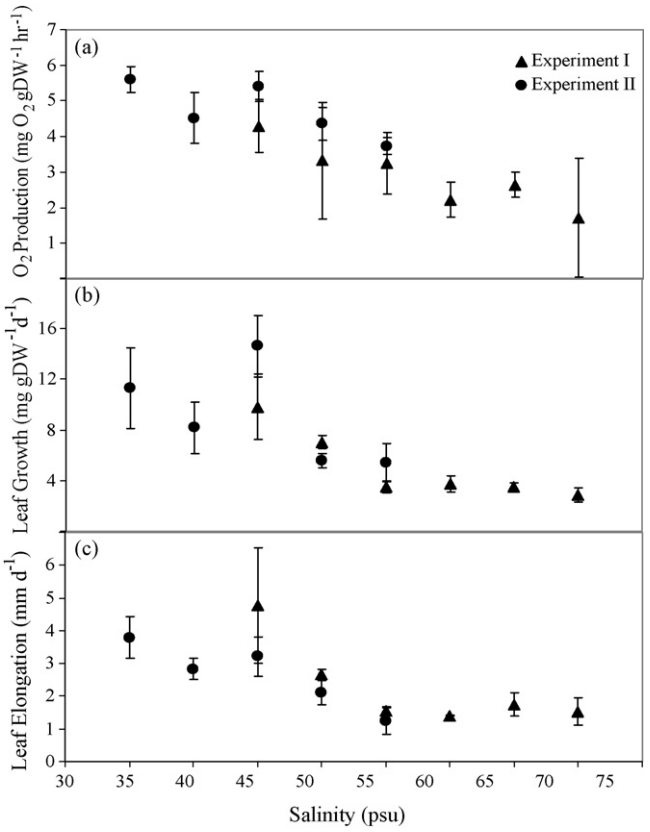

Fig. 3. *Thalassia testudinum* (a) chamber O_2 production rates, (b) growth rates as new leaf biomass normalized to photosynthetic tissue and (c) leaf elongation rates as a function of pulsed (3 days) hypersalinity treatments from Experiment I (45–70 psu) and Experiment II (35–55 psu). Means presented with S.E.

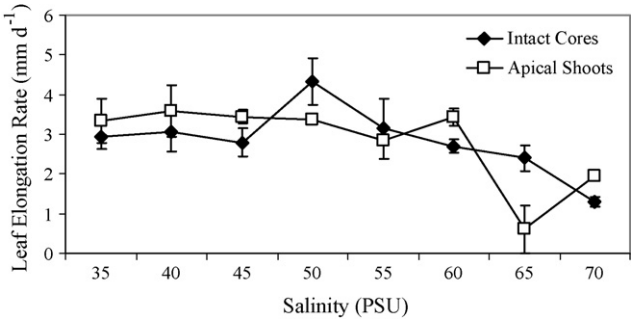

Fig. 4. *T. testudinum* leaf elongation rate (mm d^{-1}) across a range of salinities 30 days post-treatment from intact sediment cores and apical rhizome shoots (Experiment III; mean ± S.E.).

3.2. Mesocosm experiment (III): T. testudinum, H. wrightii, R. maritima

Based on the results of our hydroponic experiment and initial hypothesis, we expected *T. testudinum* to show a salinity threshold slightly above 45 psu in Experiment III, but in fact the threshold was extended up to 60 psu. Growth rates for *T. testudinum* were less than 2 mm d^{-1} only in the two highest salinity (65 and 70 psu) treatments even after 30 days of exposure (Fig. 4). Leaf quantum efficiencies were also greater than 0.7 up to 60 psu and only fell below this threshold at 70 psu (Fig. 5). No total shoot mortality was observed in any of the intact cores for *T. testudinum*, and shoot densities remained fairly stable throughout the experiment, particularly in the apical rhizome shoots (Fig. 6). Even in the intact cores, total shoot numbers only fell 25% in the 65–70 psu treatment tanks.

Comparing amongst the seagrass species examined, *H. wrightii* appeared to be the most salt tolerant, increasing or maintaining shoot numbers under all hypersaline treatments in the intact cores (Fig. 6). Shoots with apical rhizomes had a significant reduction only at 70 psu, with the exception of the 60 psu tank where we had a "blow-out" when a powersweep dislodged from the side of the tank. Shoot plastochron interval was 5–7 days per new shoot for *H. wrightii* with no clear hypersaline effect (Fig. 7). Shoot growth rates were not significantly different among salinity treatments for *H. wrightii*, but leaves were beginning to exhibit chlorosis at 70 psu (personal observation) reflected in the PAM results. Reduced quantum efficiency of photosystem II (F_v/F_m) was observed at the highest salinity treatments, although across all salinity levels F_v/F_m were still greater than 0.7 (Fig. 5).

Of the three species, *R. maritima* incurred the highest percent shoot loss in both intact and apical rhizome cores, however with high variance. In general shoot reductions were greatest beyond 55 psu and the lowest density was found at 70 psu in the intact cores (Fig. 6). None of the salinity treatments caused total shoot mortality and in fact we observed flowering shoots under all salinity treatment levels, including 70 psu. Consistent with the lack of a significant shoot density response to salinity treatments, *R. maritima* quantum efficiency showed little change as a function of salinity, remaining between 0.70 and 0.75, with only a slight decrease at 70 psu (Fig. 5) where shoot reduction was also greatest (Fig. 6). The

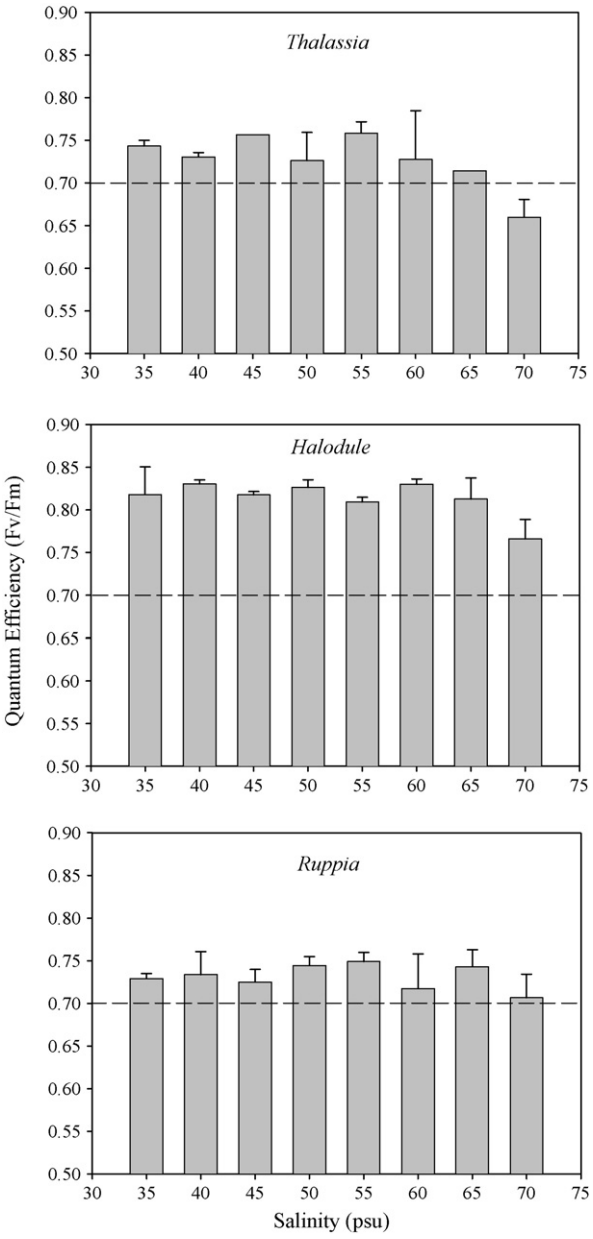

Fig. 5. Dark adapted leaf quantum efficiency as a function of salinity 30 days post-treatment for *T. testudinum*, *H. wrightii* and *R. maritima* (Experiment III; mean ± S.E.). Below the 0.70 level is considered an index of stress.

decline in shoots and high variance found for *R. maritima* is probably due to this species ephemeral nature and the fact that it tends to senesce late in the growing season when this experiment was conducted (October–November).

The high resilience of all three seagrass species to hypersalinity in the mesocosm experiment, in contrast to results from the hydroponic pulse salinity experiment for *T. testudinum*, appears to be due to their ability to osmoregulate. A slow in situ rate of salinity increase (1 psu d^{-1}) resulted in a linear correlation between leaf osmolality (mmol kg^{-1}) and salinity treatment (psu) for *T. testudinum* and *H. wrightii* (Fig. 8a). *R. maritima* leaf osmolality increased as a linear function of salinity from 50 to 65 psu then dropped at 70 psu, perhaps related to stress at this upper level (Fig. 8b). No

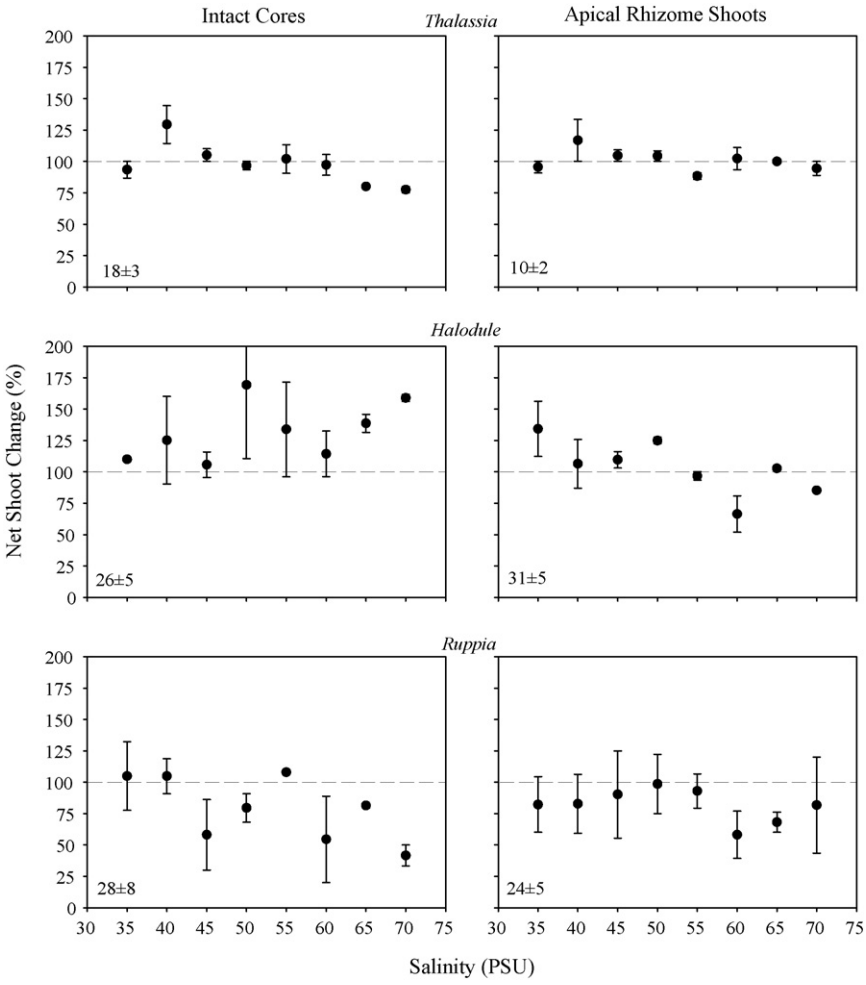

Fig. 6. Average percent change in live shoot numbers (±S.E.) in intact cores and apical rhizome shoots across all salinity levels 30 days post-treatment for *T. testudinum*, *H. wrightii* and *R. maritima* (Experiment III). The initial average shoot number for each species and experimental unit is given in the lower left corner of each graph with S.D. ($n = 16$).

consistent pattern was found across all salinities in this experiment for *R. maritima*. However, in a follow up mesocosm experiment where we examined salinity x temperature interactions we observed a highly linear relationship between salinity and osmolality (osmolality = 34 × salinity + 1566; $R^2 = 0.98$) from 37 to 60 psu at ambient temperature of 28 °C (Koch, unpublished data). In this interaction study, we also found *R. maritima* osmolality within the same range as the study reported herein.

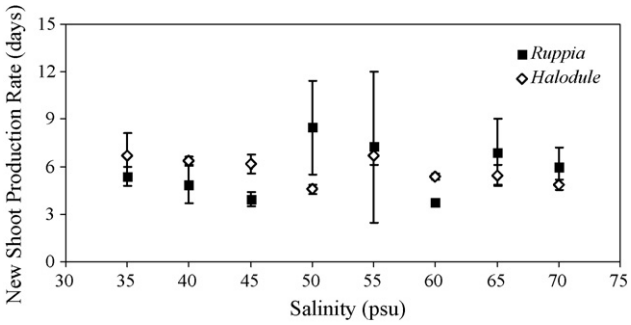

Fig. 7. Plastichrone interval (days per new shoot) for *Halodule wrightii* and *Ruppia maritima* across a range of salinities 30 days post-treatment from tagged apical rhizome shoots in Experiment III (mean ± S.E.).

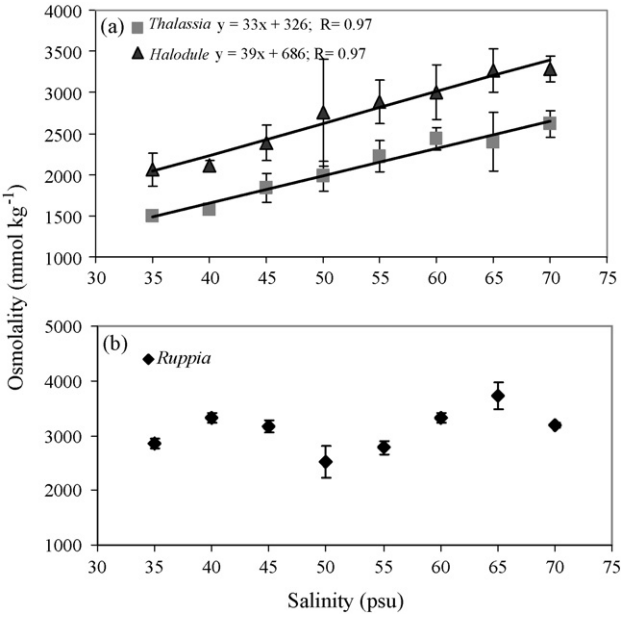

Fig. 8. Total leaf osmolality across salinity treatment levels for (a) *T. testudinum*, *H. wrightii* and (b) *R. maritima* after 30 days exposure in Experiment III. The linear fit and equation is presented for *T. testudinum* and *H. wrightii*.

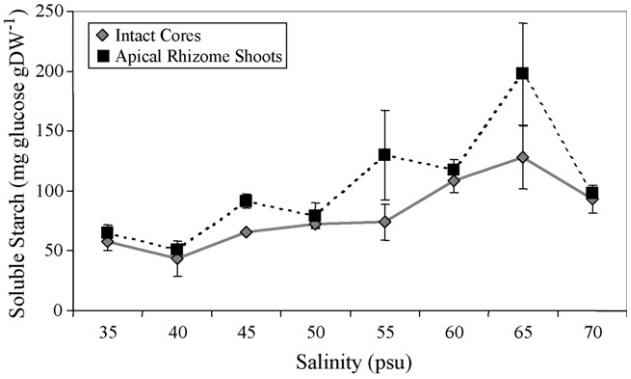

Fig. 9. Soluble starch in leaves of *T. testudinum* as a function of salinity 30 days post-treatment from intact cores and apical rhizome shoots in Experiment III.

Some of the intracellular osmotica associated with increasing salinity in *T. testudinum* leaves may have been organic sugars. Soluble carbohydrate levels in *T. testudinum* leaves in both intact and apical rhizome cores increased as a function of salinity up to 65 psu (Fig. 9) and subsequently declined, comparable to the decline in quantum efficiency at 70 psu (Fig. 6). No other consistent trends were observed for soluble carbohydrates or insoluble starch as a function of increased salinity for any other tissues in *T. testudinum* or the other two species examined.

4. Discussion

The three tropical seagrass species investigated in this study were highly tolerant of hypersaline conditions in mesocosms. None of the three species elicited total mortality of shoots across the range of salinities examined (35–70 psu) over a 30-day period of continuous exposure. Based on stress indicators, shoot decline, growth rates, and PAM florescence, all three species were able to tolerate salinities up to 55 psu, with *T. testudinum* (60 psu) and *H. wrightii* (65 psu) eliciting a slightly higher salinity threshold than *R. maritima* (55 psu). However, threshold levels of salinity tolerance dropped 20 psu to approximately 45 psu for *T. testudinum* when salinity was pulsed without a slow osmotic adjustment period. Lirman and Cropper (2003) also found *T. testudinum* to exhibit decreased leaf elongation rates and slow growth rates at 40 and 45 psu in a pulsed experiment that was designed to evaluate the effect of pulsed freshet events (5–45 psu). In addition to *T. testudinum*, variations in the upper salinity threshold have been reported for several other seagrass species related to rates of salinity increase. Based on a literature review of hypersalinity studies on seagrasses (Table 1), we found that pulsed experiments resulted in at least a 10 psu lower threshold (41 ± 3 psu) than experiments applying a gradual rate of salinity increase (57 ± 11). In pulsed experiments, the range of salinities tested tended to be lower, probably contributing the lower average thresholds; however, pulsed experiments consistently defined a low salinity threshold for seagrass (Table 1). Further, observations of seagrass species growing under relatively high salinities in the field (Table 2) support the contention that many seagrass species are quite salt tolerant, and have the ability to survive at relatively high salinities, albeit under gradual rates of salinity increase.

The precipitous decline in growth and photosynthesis that was found for *T. testudinum* (this study) and quantum yield for *R. maritima* (Murphy et al., 2003) at 45–50 psu in pulsed hypersalinity treatments suggest that a slow rate of salinity exposure is required for osmoregulation. Our data indicate that *T. testudinum* accumulates soluble sugars in its leaves in response to increasing salinity, possibly acting as an osmoticum along with the amino acid proline (Pulich, 1986), or as a consequence of increasing chloroplasts. Jagels (1973, 1983), based on his leaf ultrastructure studies, suggested that osmoregulation in *T. testudinum* is the result of anion secretion or exclusion across epidermal leaf cells promoted by a highly invaginated plasmalemma and supported by a high density of chloroplasts and mitochondria at active sites of salt transport. While this osmoregulatory role of the plasmalemma–mitochondrial system in the epidermal cells of *T. testudinum* was questioned by Iyer and Barnabas (1993), they also observed similar ultrastructural changes in *Zostera capensis* with increasing salinity: greater surface area of the plasmalemma and an increased density of chloroplasts up to 60 psu, a point at which chloroplast deformations occurred. If cellular morphological changes in addition to biochemical adaptations are required for *T. testudinum* to tolerate hypersalinity, a slow rate of salt increase that would allow plants time to acclimate, should increase their salinity tolerance threshold. Above these upper thresholds (65–70 psu) plant membranes and organelles are probably overwhelmed by ions and become denatured, as was observed in *Z. capensis* at 65 psu (Iyer and Barnabas, 1993). We also observed physiological stress in seagrasses at about 60 psu, close to the average threshold we calculated based on experiments using slow osmotic adjustment (57 psu, Table 1).

T. testudinum seedlings and *R. maritima* seed germination have also been examined for hypersalinity tolerance thresholds under both gradual and pulsed hypersalinity treatments. When exposed to pulsed salinity increases, *R. maritima* seeds germinated at a maximum of 20 psu, but were able to germinate at 28 psu when salinity was gradually increased (0.65 psu d^{-1}; Kahn and Durako, 2005). It is interesting to note that while we and others (McMillan and Moseley, 1967; see review in Kantrud, 1991) find the adult stage of *R. maritima* to be highly tolerant of high salinity and able to produce reproductive shoots with seeds across a range of seawater salinity (35–70 psu, this study), other studies have observed much lower thresholds and identified rate of salinity change or salinity variance as an important component in explaining discrepancies in salt tolerance reported for *R. maritima* (Verhoeven, 1979; La Peyre and Rowe, 2003; Kantrud, 1991). While different ecotypes could explain some of the differences in salt tolerance for adult plants (McMillan and Moseley, 1967; Kantrud, 1991), *R. maritima* only dominates at the low salinity freshwater-marine ecotone in Florida Bay, while these plants are clearly physiologically able to tolerate high salinity. Discrepancies in salinity tolerance have also been found for *T. testudinium* seedlings with and without time for

Table 1
Effects of pulsed vs. slow rates of salinity increase in defining seagrass hypersalinity thresholds (psu) by various stress response parameters and upper salinity survival limits from various seagrass genus (*Thalassia*, *Halodule*, *Ruppia*, *Zostera*, *Syringodium*, *Amphibolis*, *Posidonia*, *Halophila*)

Species	Threshold	Range	Survival	Rate	Parameters	Reference
I. Pulsed experiments						
T. testudinum	40	5–45	45	Pulsed	Leaf elongation	Lirman and Cropper (2003)
T. testudinum	40	0–70	50	Pulsed	Seedling growth, survival	Kahn and Durako (2006)
T. testudinum	45	36–70	70	Pulsed	Leaf growth, O_2 prod.	This study
Halodule wrightii	35	5–45	45	Pulsed	Leaf elongation	Lirman and Cropper (2003)
R. maritima	40	0–40	40	Pulsed	Quantum yield, osmolality	Murphy et al. (2003)
S. filiforme	40	5–45	45	Pulsed	Leaf elongation	Lirman and Cropper (2003)
A. Antarctica	42.5	35–65	57.5	Pulsed	Seedling root production, survival	Walker and McComb (1990)
P. oceanica	42	25–57	<50	Pulsed	Growth, mortality	Fernandez-Torquemada and Sanchez-Lizaso (2005)
Average	41 ± 3					
II. Slow rate salinity increase						
T. testudinum	60	28–74	74	0.75 psu d^{-1}	Growth, chlorophyll content	McMillan and Moseley (1967) (*H. wrightii* as *Diplanthera*)
T. testudinum	50	0–70	70	0.66 psu d^{-1}	Seedling growth, survival	Kahn and Durako (2006)
T. testudinum	60	36–70	70	1.0 psu d^{-1}	Growth, quantum yield, O_2 prod.	This study
H. wrightii	65	36–70	70	1.0 psu d^{-1}	Growth, osmolality, quantum yield	This study
H. wrightii	70	28–74	74	0.75 psu d^{-1}	Growth, chlorophyll content	McMillan and Moseley (1967) (*H. wrightii* as *Diplanthera*)
R. maritima	70	28–74	74	0.75 psu d^{-1}	Growth, chlorophyll content	McMillan and Moseley (1967) (*H. wrightii* as *Diplanthera*)
R. maritima	55	36–70	70	1.0 psu d^{-1}	Growth, osmolality, quantum yield	This study
S. filiforme	45	28–74	60	0.75 psu d^{-1}	Growth, chlorophyll content	McMillan and Moseley (1967) (*H. wrightii* as *Diplanthera*)
Z. capensis	40	0–60	60	1–2 psu $7d^{-1}$	Ultrastructural changes, growth	Iyer and Barnabas (1993)
Average	57 ± 11					
III. Moderate rate salinity increase						
R. cirrhosa	55	0–75	75	5.0 psu d^{-1}	Flowering, biomass, growth	Adams and Bate (1994)
Z. capensis	35	0–75	55	7.0 psu d^{-1}	Rhizome and leaf biomass, growth	Adams and Bate (1994)
Halophila johnsonii	30	0–60	50	10 psu d^{-1}	Growth, photosynthesis	Fernandez-Torquemada et al. (2005)

osmotic adjustment. Kahn and Durako (2006) found *T. testudinum* seedlings to survive at 20, 30, and 40 psu in pulsed hypersalinity treatments and no survival at 50 psu. However, when exposed to slower increases in salinity (0.66 psu d^{-1}) all seedlings survived at 50 psu.

Thus, seagrass in the adult form as well as seeds and seedlings show different thresholds to hypersalinity based on the rate of salinity increase. Under pulsed hypersaline conditions, such as in the effluent discharge of reverse osmosis operations, we predict that seagrass species would have a

Table 2
Upper ranges of salinities where seagrasses have been observed growing in the field under hypersaline conditions

Species	Salinity range (psu)	Location and conditions	Reference
Thalassia testudinum	15–40	2 year (1969–1970) *T. testudinum* beds, Biscayne Bay, FL	Zieman (1975)
T. testudinum	50–>60	Chronic hypersaline period (1989–1990), central Florida Bay, FL	Zieman et al. (1999)
T. testudinum	28–54	Hypersaline conditions (2004–2005), Florida Bay, FL	Koch (unpublished data)
Halodule wrightii	35–62	Extensive beds, salinities recorded 1996–1997, Baffin Bay, TX	Cotner et al. (2004)
Amphibolis antarctica	35–62.4	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Halodule uninervis	35–64	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Halophila ovalis	35–55	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Halophila ovata	35–55	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Posidonia australis	35–55	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Posidonia coriacea	35–50	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Cymodocea augustata	38–50	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Halophila spinulosa	35–48	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Syringodium isoetifolium	35–45	Adult plants infrequent above this range, Shark Bay, Australia	Walker et al. (1988)
Ruppia maritima	0–>60	A very wide range of salinities observed for this species based on review	Kantrud (1991)

significantly lower salt threshold compared to those in evaporative basins. Tropical species that have evolved under intermittent or chronic exposure to hypersalinity, where salinity is gradually increased through evaporative processes, should have higher salinity tolerances. However, tropical species are living at the edge of their upper physiological limits of salinity (Walker, 1985; Walker et al., 1988, this study) and temperature (Zieman, 1975, Koch unpublished data), so further increases in salinity as a result of climate change and freshwater extraction may have significant consequences for tropical seagrasses, particularly in estuaries with restricted circulation and high rates of evaporation such as Shark Bay, Baffin Bay, and Florida Bay.

Hypersalinity has been hypothesized to play a role in large scale (4000 ha 1987) and recurrent small scale mortality of seagrass beds in Florida Bay (Robblee et al., 1991; Fourqurean and Robblee, 1999; Zieman et al., 1999). The results of this study do not support the hypothesis that sudden large-scale dieoffs of seagrass in the Bay can be solely explained by moderate hypersalinity exposure, also articulated by Zieman et al. (1999). Our experiments and others (Table 1) indicate that tropical seagrass species are highly tolerant of short-term exposure to moderate hypersaline conditions with slow rates of salinity increase. However, secondary stressors associated with hypersaline conditions, such as excessive carbon drain associated with osmoregulation through the production of compatible solutes (Flowers et al., 1977; Van Diggelen et al., 1987; Pulich, 1986; Murphy et al., 2003) and increased O_2 consumption to meet ATP respiratory requirements for active ion transport systems (Jagels, 1983), can decrease the plants overall fitness and resilience to stress, particularly in locations with a high sediment O_2 demand as is the case in western Florida Bay (Carlson et al., 1994; Koch unpublished data). Other stressors that co-occur in the field with high salinity, such as high temperature and porewater sulfides, can influence photosynthesis and O_2 consumption rates in seagrass communities (Zieman, 1975; Kerr and Strother, 1985; Carlson et al., 1994; Koch and Erskine, 2001; Borum et al., 2005; Koch unpublished data). Thus, while we found all three Florida Bay seagrass species to be highly tolerant of hypersalinity over a 30-day exposure period in mesocosms, and conclude that hypersalinity probably does not in itself cause seagrass dieoff events in the Bay, synergistic effects may be important (Koch and Erskine, 2001). In addition, long term exposure to moderate hypersaline conditions could be a cumulative stress. Historical data from Florida Bay show that hypersaline conditions (>35 psu) can perpetuate for over a year or more (Fig. 1) instilling a chronic stress which could influence the long-term health of the seagrass community. A chronic salinity stress is found in Shark Bay bottom waters which results in the dominant seagrass *Amphibolis antarctica* having a high specific growth rate between 43 and 50 psu, and maximum growth and biomass at 43 psu (Walker and McComb, 1990). With the exception of *A. antarctica* and *H. uninervis*, the majority of seagrass species (12 total) in Shark Bay are restricted to areas with salinities ≤55 psu (Walker et al., 1988). The impact of seagrass chronic exposure to hypersaline conditions extending over multiple years requires further evaluation, particularly in light of increasing ocean temperatures resulting from climate change.

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