Plasticity in turtle grass (*Thalassia testudinum*) flower production as a response to porewater nitrogen availability

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Abstract

Terrestrial plants often demonstrate plasticity in reproductive timing and output in response to environmental conditions such as temperature, photoperiod, water availability and nutrients. Despite the importance of sexual reproduction for seagrass establishment and persistence, factors influencing reproductive timing and output of these underwater marine plants remain largely unknown. We used a manipulative field-based experiment to assess the effect of porewater nitrogen on turtle grass (*Thalassia testudinum*) flower production. Experiments were conducted within monospecific turtle grass beds in Lower Laguna Madre, Texas, a region with consistently low water column and porewater nutrient levels. We enriched 50 turtle grass plots with fertilizer buried within the sediment at the rhizome layer. This resulted in increased porewater ammonium concentrations of 679 ± 188 μM in enriched plots, compared to 204 ± 34 μM in unenriched plots. After the onset of the reproductive season, we examined turtle grass reproductive status, plant morphology and elemental composition. Unenriched plots had a higher proportion of reproductive shoots (0.19 ± 0.10) than enriched (0.08 ± 0.04) plots. Shoots from enriched plots, alternatively, assimilated additional available nitrogen into leaf tissue and had more leaves that were longer and wider than their unenriched counterparts. Our results indicate that turtle grass exhibits plasticity in reproduction as a response to nutrient availability, whereby under low porewater nitrogen conditions, resources are diverted to sexual reproduction rather than somatic growth. The worldwide increase in coastal nutrient loading, particularly in the form of submarine groundwater discharge, has the potential to reduce flowering in *Thalassia* species.

Keywords: seagrass; turtle grass; nutrient; flowering; reproduction; somatic growth

1. Introduction

Sexual reproduction is energetically costly and, as a result, reproductive output often varies over space and time (Obeso 2002). For many plants, the allocation of resources to reproduction and somatic growth is related to environmental conditions such as temperature, photoperiod, water availability and nutrients (Wada and Takeno 2010). This plasticity in reproduction is common and especially pronounced when resources are limiting (Harper 1977).

Nutrients such as nitrogen and phosphorus are essential for plant growth and are heterogeneous within the soil over fine spatial scales (Larcher 1975). Their availability can influence plant morphology, production, and ecological interactions such as herbivory and competition (Larcher 1975; Gao et al. 2013). In many species of terrestrial herbaceous plants, nutrient availability also influences resource allocation to reproductive modes (Doust and Doust 1988); whereas some plants induce sexual reproduction under high nutrient conditions (Campbell and Halama 1993), others do so when exposed to stressfully low nutrient levels (stress-induced flowering; Wada et al. 2010). For example, two eudicots, *Pharbitis nil* and *Perilla frutescens* var. *crispa,* flower in response to poor nutrition (Wada and Takeno 2010), and flowering in *Arabidopsis thaliana* is triggered by low nitrate (NO_3^-) levels (Marin et al. 2011). Although the influence of nutrients on terrestrial plant reproductive allocation has been widely studied, the effect of nutrient availability on reproductive allocation in their marine counterparts (seagrasses) is unknown.

Seagrasses are a widespread group of over 70 species of submerged marine plants that, unlike terrestrial plants, can assimilate inorganic nutrients through both above-ground (leaf) and below-ground (root) tissues. The complex mechanisms of uptake and assimilation of porewater and water column nutrients by seagrasses have been studied at length (Duarte 1990; Lee and

Dunton 1999), and as a result, it is well known that nutrient availability affects most aspects of seagrass biology, physiology and ecology (Armitage et al. 2005). Seagrass growth is often nutrient-limited, as studies enriching porewater and/or water column nutrients have shown marked increases in growth and changes in morphology (Duarte 1990; Ferdie and Fourqurean 2004). The exact nutrient limiting seagrass growth can be species-, location- and/or time-dependent (Fourqurean et al. 1992), but the most common limiting nutrients are nitrogen or phosphorus (Duarte 1990). Worldwide increases in anthropogenic nutrient loading and eutrophication are exposing seagrasses to higher than normal nutrient levels (Nixon 1995), and though it is expected that this increased nutrient loading will alter seagrass growth, productivity, and ecology (Burkholder et al. 2007), the effects on seagrass reproduction remain unclear.

As angiosperms, seagrasses have the ability to reproduce sexually and also propagate clonally through lateral rhizome growth. Historically, sexual reproduction was considered rare for seagrass genera (den Hartog 1970) and this expected rarity led to a dominance of literature examining clonal growth (Hemminga and Duarte 2000). However, recent research indicates that sexual reproduction is important for both seagrass bed establishment and maintenance (van Dijk et al. 2009). The limited historical research on seagrass reproduction leaves many questions unanswered, specifically concerning environmental factors that influence reproductive output and the relative importance of vegetative versus sexual reproduction for individual species.

Turtle grass (*Thalassia testudinum* Bank ex König) is a dominant seagrass species throughout the Gulf of Mexico and Caribbean and is usually located in areas with low water column ($PO_4^{3-} < 1$ μM and $NH_4^+ + NO_3^- < 3$ μM; van Tussenbroek et al. 2006) and porewater (ammonium, NH_4^+ between 2–200 μM, Lee and Dunton 1999) nutrients. Nutrient uptake occurs through both the leaves and roots (Lee and Dunton 1999) and nutrient addition experiments have

resulted in increased somatic growth and production (Lee and Dunton 1999a). As with all seagrass species, turtle grass expands clonally by horizontal rhizome extension and also reproduces sexually. Horizontal propagation is relatively slow, with rhizome extension between 19–35 cm $year^{-1}$ $apex^{-1}$ (Gallegos et al. 1992; van Tussenbroek 1998). Turtle grass plants are dioecious (i.e. each clone is individually male or female), but clones of both sexes often grow intermixed (van Tussenbroek et al. 2006). The turtle grass reproductive season varies along the species range, but generally spans summertime months (van Tussenbroek et al. 2006). During the reproductive season, flowers are produced near the sediment at the base of the shoot, and upon successful pollination of the female flower, a fruit is produced. Fruits contain 1–6 seeds, but most commonly have two (Kaldy and Dunton 2000; van Tussenbroek et al. 2010). Although several recent studies have investigated the reproductive ecology of turtle grass (e.g. Kaldy and Dunton 2000; van Tussenbroek et al. 2008, 2012; van Tussenbroek and Muhlia-Montero 2013), factors regulating flowering and reproductive output are not fully understood.

Here, we present the first measurements directed at how nutrient availability influences turtle grass flowering and reproductive output. Specifically, we determined the influence of porewater and assimilated nutrients on: 1) turtle grass flower production and 2) turtle grass somatic (leaf) growth. We hypothesized that: 1) turtle grass demonstrates reproductive plasticity in response to porewater nutrient availability, and 2) flower production and somatic growth are inversely related. We conducted a nutrient enrichment experiment in turtle grass-dominated beds in south Texas and evaluated the reproductive status and somatic growth of turtle grass relative to porewater ammonium levels and leaf elemental composition (e.g. carbon, nitrogen and phosphorus levels and molar ratios).

2. Materials and methods

2.1 Study sites

Nutrient enrichment experiments were conducted in Lower Laguna Madre, Texas. This area has been the site of several prior studies investigating seagrass biology (Lee and Dunton 1999, 1999a) and is characterized by consistently low dissolved inorganic-N reflected in both porewater (~30 μM porewater NH_4^+, Lee and Dunton 1999a) and water-column (~1 μM water-column NH_4^+, NO_3^-, and NO_2^-, Lee and Dunton 1999a) nutrient levels. Sediments in the study area are composed primarily of sand, with contributions of silt, clay and rubble (Kaldy and Dunton 2000). Five sites (each 150 m × 150 m) were selected, each of similar depth (122 ± 5.33 cm) and characterized by monotypic turtle grass meadows. Within each of the five sites, ten stations were randomly chosen (n= 50 stations total) with at least 15 m between stations to avoid re-sampling individual genets (van Dijk and van Tussenbroek 2010). Each station contained paired unenriched and enriched plots (see below).

Environmental parameters (water temperature, salinity, pH) were measured at the beginning (29-Mar-2012), middle (26-Apr-2012) and end (22-May-2012) of the experiment with a YSI 600XL data sonde. Measurements were taken at the top of the seagrass canopy in each of the five sites at the center of the ten stations.

2.2 Nutrient enrichment experimental design

Approximately 2 months before onset of the reproductive season in Lower Laguna Madre (Spring, K. Darnell, pers. obs), two 0.25 m^2 plots were established at each station. One plot served as a control (unenriched plot) and the other plot was enriched with Osmocote Smart-Release® Plant Fertilizer (N-P-K 19:6:12; enriched plot). In the enriched plots, 30.375 g of

137 fertilizer pellets were wrapped in cheesecloth to obtain the manufacturer's suggested application
138 dosage and buried ~10 cm below the sediment surface at the rhizome layer in the center of the
139 plot (Lee and Dunton 1999a). Based on the manufacturer's values, the amount of N and P
140 applied to each 0.25 m^2 enriched plot was 5.77 g N and 1.8 g P. Unlike aboveground fertilizer
141 application, belowground fertilizer application eliminates potential confounding effects of
142 increased leaf epiphyte cover (Lee and Dunton 1999a). A third plot was added to a random
143 subset of stations (n = 2 stations per site) to test for effects of cheesecloth bag burial. These plots
144 served as "empty bag control" plots. In these plots, an empty cheesecloth bag was buried
145 belowground at the rhizome layer in the center of the plot. Rhizomes were severed along the
146 perimeter of each plot to a depth of ~30 cm to avoid potential translocation of nutrients (Lee and
147 Dunton 1999a).

148 The experiment was initiated on 29-Mar-2012. At the time of plot establishment, two
149 replicate sediment samples were collected from within 10-cm of the center of each unenriched,
150 enriched and control plot at a subset of stations (n=2 stations per site) with an 80 mL syringe for
151 analysis of sediment porewater NH_4^+. Sediment porewater was obtained by centrifugation (5000
152 x *g* for 20 minutes) and NH_4^+ content was analyzed using standard colorimetric techniques
153 following Parsons et al. (1984). Three intact turtle grass shoots consisting of all aboveground and
154 belowground tissue were also collected from within 10-cm of the center of each plot for
155 assessment of seagrass reproductive status, morphology, and leaf nutrient content (see below).

156 The experiment concluded on 22-May-2012, 54 d after experimental plots were
157 established. At the conclusion of the experiment, two replicate sediment samples were again
158 collected from within 10-cm of the center of each plot at a subset of stations (n=2 stations per
159 site) and analyzed for sediment porewater NH_4^+ content. Fertilizer bags were also collected from

each enriched plot, re-weighed, and daily nitrogen delivery was estimated. Five intact turtle grass shoots were collected from within 10-cm of the center of each unenriched, enriched and control plot for assessment of seagrass reproductive status, morphology, age, and leaf nutrient content. Previous studies indicate that a 2-month duration is sufficient for sediment nutrient enrichment and seagrass assimilation (Lee and Dunton 2000) and for observing changes in plant reproductive output (Johnson and Decoteau 1996).

2.3 Seagrass reproductive status, morphology, age and historic flowering

Each shoot collected at the beginning and end of the experiment was assessed for reproductive status by inspecting for the presence of reproductive tissues (e.g. developing flowers or fruits). For March and May, area of the longest leaf (cm^2) was calculated for each shoot by multiplying the length (cm) and width (cm) of the longest leaf on that shoot. For May samples only, the aboveground dry weight (g) of each shoot was determined by scraping the leaves free of epiphytes and drying at 60 °C to a constant weight. Also for samples collected in May, to assess for potential confounding factors, the dry weight of epiphytes on leaf tissue was obtained for a subset (n=130) of these shoots, shoot age was estimated by counting the number of leaf scars on the vertical rhizome and dividing by the annual leaf production (formation) rate for turtle grass in this area (13 leaves $year^{-1}$, Kaldy et al. 1999), and the number of flower scars was also counted on each shoot to reconstruct historic (pre-experiment) flowering frequency of shoots from unenriched and enriched plots.

2.4 Leaf tissue nutrient analyses

Preliminary analyses of replicate shoots from the same plot indicate that variance (s^2) in carbon, nitrogen and phospohorus levels within the plots was low (%C: 1.00 ± 0.286, %N: 0.041 ± 0.011, %P: 0.0009 ± 0.0002). As a result, leaf tissue carbon, nitrogen, and phosphorus were measured in both March and May in one randomly chosen shoot collected from within 10-cm of the center of each plot. Shoots were rinsed, wiped free of epiphytes and dried at 60°C to a constant weight before being ground to a fine powder using a Wig-L-Bug® grinding mill. Ground tissue was sent to the University of California at Davis Stable Isotope Facility for analysis of total carbon and total nitrogen using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were analyzed for total phosphorus at the University of Texas Marine Science Institute on a Shimadzu UV-2401 PC UV-VIS Recording Spectrophotometer following a modified protocol from Chapman and Pratt (1961). Carbon, nitrogen and phosphorus data were used to calculate %C, %N and %P and molar C:N:P ratios.

2.5 Statistical analyses

For samples collected in May comparing plots enriched and unenriched with fertilizer, porewater NH_4^+, proportion C, proportion N, proportion P, and epiphyte biomass were analyzed using analysis of variance (ANOVA) with treatment as a fixed factor and site and station (nested within site) as random factors. Residuals were normally distributed for these variables. To obtain normally distributed residuals of the other variables, the measured values were transformed before analysis with ANOVA. The square-root of leaf area, the square-root of dry mass, the

square-root of shoot age, and the $\log_{10}$ of historic flowering, C:N, of C:P, and of N:P were analyzed.

Samples collected in May comparing empty bag control plots and unenriched plots were also analyzed using ANOVA with treatment as a fixed factor and site and station (nested within site) as random factors. For these analyses, area of the longest leaf and leaf dry weight were square root transformed, proportion P was arcsine transformed and proportion N and porewater NH_4^+ were $\log_{10}$ transformed to achieve normality of the residuals. Residuals of proportion C, C:N, C:P and N:P were normally distributed.

Samples collected in March (before the initiation of the experiment) comparing plots enriched and unenriched were similarly analyzed using ANOVA with treatment as a fixed factor and site and station (nested within site) as random factors. Residuals of proportion C, proportion N and N:P were normally distributed. To obtain normally distributed residuals of some of other variables, we transformed the measured values before analysis with ANOVA. The $\log_{10}$ of porewater NH_4^+, proportion P, C:N, C:P and area of the longest leaf were analyzed.

Reproductive status (whether a shoot was reproductive or not) was analyzed with a generalized linear model with a binomial distribution and a logit link function, using the GLIMMIX procedure in SAS v. 9.4 (SAS Institute Inc., Cary, NC). Treatment was a fixed factor and both site and station (nested with site) were random factors. Data are presented as the proportion of shoots that were reproductive.

Unless otherwise specified, all analyses were conducted in JMP v. 11 (SAS Institute Inc., Cary, NC) and data are presented as the mean ± standard error (mean ± S.E.).

3. Results

In March, prior to the initiation of the experiment, enriched and unenriched plots did not differ for any of the variables measured (Table 1). In May, there were no differences between unenriched and control (empty bag control) plots, indicating that cheesecloth bag burial did not influence results with respect to disturbance of below-ground tissues, and that there was no fertilizer contamination into the unenriched plots (Table 2).

3.1 Environmental parameters

Water temperature increased throughout the experiment from 25.3 ± 0.4 °C in March to 25.6 ± 0.3 in April, and 27.0 ± 0.6 °C in May. All sites remained saline throughout the experiment (March: 32.8 ± 0.5, April: 30.1 ± 0.2, May: 34.6 ± 0.6), and pH displayed low spatial and temporal variability (March: 8.00 ± 0.06, April: 8.33 ± 0.11, May: 7.91 ± 0.02).

3.2 Porewater NH_4^+

At the beginning of the experiment, prior to nutrient enrichment, sediment porewater NH_4^+ levels were initially relatively low in both the unenriched (198.9 ± 33.9 μM) and enriched (154.0 ± 17.8 μM) plots ($P = 0.06$; Figure 1). Fertilizer bag weight decreased by 62.3 ± 1.5% over the course of the experiment, delivering 0.34 ± 0.01 g fertilizer day^{-1} to the enriched plots. As a result, porewater NH_4^+ levels were elevated in the enriched plots (679.9 ± 188.1 μM) compared to the unenriched plots (203.6 ± 34.7 μM; $P < 0.002$) by the conclusion of the experiment in late May (Figure 1).

3.3 Shoot reproductive status, morphology, age, and historic flowering

All shoots were non-reproductive in March, confirming that the experiment began before the onset of the turtle grass reproductive season in this area. In May, 18.9 % of the shoots were reproductive. Of the reproductive shoots, 62 had developing flowers and 27 bore fruit. Unenriched plots had over double the proportion of reproductive shoots (0.19 ± 0.10) than enriched plots (0.08 ± 0.04) ($P = 0.0002$) (Figure 2a). Shoots in enriched plots, however, had more aboveground biomass ($P < 0.0001$, Figure 2b) and a greater longest leaf area ($p = 0.0003$, Figure 2c) compared to unenriched shoots. Epiphyte dry weight was similar for shoots from unenriched and enriched plots ($P = 0.22$). Age did not differ between unenriched and enriched shoots ($P = 0.44$, Figure 3a), shoot ages ranged from 1–10 years with 97% of shoots between 1 – 6 years, and most (96%) of the reproductive shoots were between ages 2 and 6 years (Figure 3b). The number of historic (pre-experiment) flower scars did not differ between unenriched and enriched shoots ($P = 0.52$).

3.4 Leaf tissue carbon, nitrogen and phosphorus content

Leaf tissue carbon (%C), nitrogen (%N), phosphorus (%P) and molar C:N:P ratios did not differ between unenriched and enriched plots at the beginning of the experiment (March, Table 1) and all nutrient levels were within the reported range for turtle grass (Duarte 1990). At the conclusion of the experiment in May, shoots collected from the enriched plots had significantly higher leaf tissue %C ($p = 0.008$) and %N ($P < 0.0001$) than shoots from unenriched plots (Table 3). Phosphorus content, however, was similar between enriched and unenriched shoots ($P = 0.84$) (Table 3). Enriched shoots had lower molar C:N ($P < 0.0001$) and

higher molar N:P ($P < 0.0001$) than unenriched shoots, reflecting the elevated leaf tissue nitrogen in enriched plots. Molar C:P did not differ between treatments ($P = 0.74$) (Table 3).

4. Discussion

Our results demonstrate that in Lower Laguna Madre, Texas, turtle grass (*Thalassia testudinum*) produces more flowers under low nitrogen conditions (porewater NH_4^+ 203.6 ± 34.7 μM), than under high nitrogen conditions (porewater NH_4^+ 679.9 ± 188.1 μM). When exposed to high nitrogen, turtle grass produces fewer flowers, but increases somatic growth of aboveground leaf tissue, with shoots having more leaves that are longer and wider than their unenriched conspecifics. Although observational studies have suggested a connection between nutrients and seagrass reproduction (e.g. Short 1983, van Dijk and van Tussenbroek 2010), to our knowledge this is the first direct experimental evidence of plasticity in flower production in response to in situ porewater nitrogen concentrations. These results suggest that the increase in anthropogenic nutrient loading to coastal systems (Nixon 1995), particularly in the form of groundwater nitrogen delivery, is likely to reduce turtle grass flower production and could have dramatic population level consequences for this foundation species.

4.1 Stress-induced flowering

Stress-induced flowering as a result of sub-optimal conditions is a common resource allocation strategy for terrestrial and aquatic angiosperms because of the plants' inability to physically escape poor surroundings (Wada and Takeno 2010). Inducing sexual reproduction under sub-optimal conditions increases (1) the likelihood that recruitment of offspring to the

population will occur in the next, possibly more favorable season, and (2) the potential for offspring to escape (disperse) from the stressful conditions near the parent (Williams 1975).

Long-range propagule dissemination under nutrient limitation, resulting from stress-induced flowering, is appropriate for species that have large ranges in distribution and have the capacity for long-distance dispersal. Aquatic vascular plants often display broader distribution ranges than terrestrial plants (Santamaria 2002), and have the capacity for long-distance dispersal that can be biotically or abiotically mediated. Turtle grass fruits and seeds have the capability to disperse long distances from the parent plant and, therefore, from local environmental conditions. Buoyant fruits often detach from the parent plant where they float to the surface and are transported by currents up to 360 km (van Dijk et al. 2009). Taking into account local current and wave conditions in Lower Laguna Madre, Texas where our experiment was conducted, Kaldy and Dunton (1999) estimated that turtle grass fruits disperse up 15 km from the parent plant before the negatively buoyant seedlings are released and settle to the substrate. In a recent study examining turtle grass clonal diversity in the Caribbean, van Dijk and van Tussenbroek (2010) reported that plants from high nutrient lagoons displayed a lower reproductive frequency than plants from areas exposed to low nutrients, and Santamaria et al. (1995) reported that a species of *Ruppia* species, one of the most geographically widespread genera, stimulates flowering and produces more flowers in nutrient-poor sediments than nutrient-replete sediments. Further, in eelgrass (*Zostera marina*), a widely distributed seagrass species with a similar potential for long distance seed dispersal (up to 150 km, Harwell and Orth 2002) as turtle grass, Short (1983) reported that flowering was inversely correlated with porewater NH_4^+ concentration. However, Short (1983) sampled eelgrass in in Izembek Lagoon, AK along a nutrient gradient, but other factors such as water depth and light intensity confounded his results. Our experimental

study with turtle grass provides evidence that the patterns observed by van Dijk and van Tussenbroek (2010) and Short (1983) may indeed be a direct effect of porewater NH_4^+ concentration, suggesting that the pattern we observed with turtle grass may be common among many seagrass genera.

Although turtle grass seeds have the potential for long distance dispersal by current-mediated transport of buoyant fruits, seeds are also released while the fruit it still attached to the parent plant (van Tussenbroek et al 2010). This strategy would dramatically reduce seed dispersal distance and likely eliminate any spatial escape from local stressful conditions.

Stress-induced flowering has been reported for several seagrass species as a response to sub-optimal conditions. For example, *Cymodocea nodosa* in the northwest Mediterranean increases flowering frequency when stressed by subaqueous dune migration (Marba and Duarte 1995), and in a recent meta-analysis, Cabaco and Santos (2012) reported that in 72% of cases, seagrass reproductive effort increased with disturbances such as mechanical damage, hydrodynamic stress, and effects associated with eutrophication. Specifically for the species examined here, turtle grass, Gallegos et al. (1992) reported increased flowering in the Mexican Caribbean in response to disturbance by Hurricane Gilbert. Our results of increased reproduction under sub-optimally low nutrient conditions, reinforced by the similarity in number of historic (pre-experiment) flower scars on shoots from unenriched and enriched plots, are further supported by reports of smaller turtle grass genets (indicating more sexual reproduction) in oligotrophic areas compared to larger genets in eutrophic areas (van Dijk and van Tussenbroek 2010).

336

337 *4.2 Nitrogen limitation*

338 Turtle grass generally grows in areas with low ambient porewater NH_4^+ levels and
339 receives most of its nitrogen from these below-ground pools (Fourqurean et al. 1992). Porewater
340 NH_4^+ levels from our study are within the range reported in turtle grass meadows, and the levels
341 in our enriched treatment (679.9 ± 188.1 μM) are considered high for turtle grass beds
342 (Fourqurean et al. 1992, Lee and Dunton 1999). The tissue levels of N and P in unenriched turtle
343 grass from our study are similar to levels reported previously for this low nutrient area (Lee and
344 Dunton 1999a, Kaldy and Dunton 2000). Although the ambient porewater NH_4^+ levels at the
345 beginning of our experiment and in the unenriched plots at the end of the experiment are higher
346 than the worldwide average for seagrass beds (mean: 86 μM NH_4^+, Hemminga 1998), it appears
347 that turtle grass in our study was nitrogen-limited. Duarte (1990) suggested that plants with less
348 than 1.8% N and a C:N ratio of 19.75:1 are nitrogen limited, but despite a leaf tissue %N of 2.4%
349 and a C:N ratio of 18:1, turtle grass in this study assimilated the available nitrogen in the
350 enriched treatment and shoots increased somatic growth. Duarte (1990) also suggested that
351 plants with less than 0.20 %P and a greater C:P ratio than 474:1 are phosphorus-limited. Turtle
352 grass shoots from our site were below the %P (0.18%) and above the C:P (575:1) thresholds, but
353 did not assimilate excess P in the enriched treatment. Although inorganic phosphorus readily
354 binds to carbonate sediments, and can induce P-limitation (Short 1987), as demonstrated in
355 carbonate sediments within Florida Bay (Fourqurean et al. 1992), the sandy sediments at our
356 sites make it unlikely that the excess P was unavailable to the plants, supporting our conclusion
357 that the lack of assimilation was physiologically rather than environmentally dictated. These data

358 highlight the need to assess nutrient limitation based on experimental evidence rather than
359 nutrient content and molar ratios alone.
360 Seagrass nutrient requirements are species-specific. In Florida Bay, *H. wrightii* has a 2.6
361 and nearly 5-fold higher N- and P- demand than turtle grass (Fourqurean et al. 1992). As a result,
362 reproductive responses to nutrient conditions are also likely species-specific, and different
363 nutrient levels or thresholds may be necessary for species to exhibit reproductive plasticity. Our
364 in situ experiment addressed reproductive output in turtle grass under natural and nutrient
365 amended conditions equivalent to a three-fold increase in porewater ammonium. Consequently,
366 we are unable to determine if a threshold nutrient level or ratio exists whereby the plant switches
367 resource allocation from reproductive tissues to somatic growth. Such information would provide
368 a much more precise physiological understanding of plant reproductive strategies under an
369 experimental gradient of porewater NH_4^+ levels.
370
371 *4.3 Coastal nutrient loading*
372 The worldwide increase in coastal nutrient loading, often resulting from surface runoff,
373 has been implicated in seagrass die-offs in many areas (Burkholder et al. 2007). To avoid
374 confounding factors that accompany water column fertilization (e.g. epiphyte accumulation and
375 light limitation) and test only the effect of nutrients on turtle grass reproduction, we injected
376 fertilizer directly into the sediment for uptake by below-ground tissues. Although results from
377 this study may not be directly applicable to areas with water column nutrient loading (because of
378 the confounding factors mentioned above), we can nevertheless conclude that turtle grass is less
379 reproductive under elevated porewater NH_4^+ conditions. Our results are directly applicable to
380 areas that receive submarine groundwater discharge (SGD). Reports of nutrient loading via SGD

are becoming increasingly common and can represent a substantial localized source of nutrients (Moore 1996). Several studies have reported that seagrasses readily assimilate nutrients from SGD, and these nutrients regulate seagrass distribution and increase growth. Mutchler et al. (2007) and Peterson et al. (2012) reported that turtle grass assimilated terrestrial-derived groundwater nutrients along the Yucatán Peninsula and in Jamaica, respectively, and Carruthers et al. (2005) reported turtle grass assimilation of wastewater nitrogen from submarine spring water in Panama. Additionally, Kamermans et al. (2002) concluded that nutrient-rich groundwater intrusion influenced diversity and abundance of *Thalassia hemprichii*, a closely related and morphologically similar 'twin species' to turtle grass found throughout the western Pacific and Indian Ocean. Both *Thalassia* species support diverse assemblages of fauna and micro- and macroalgae and likely cover hundreds of thousands of square kilometers worldwide (van Tussenbroek et al. 2006). Although studies examining sexual reproduction in *T. hemprichii* are limited, the importance of this reproductive mode for meadow establishment and maintenance is recognized (Rollon et al. 2001), and a potential reduction in flowering of *Thalassia* spp. with increased nutrients could have substantial worldwide implications. Given the results of our study, it is possible that increases in nutrient-rich SGD could reduce flowering in *Thalassia* species.

4.4 Conclusion

This study examined the effects of porewater nitrogen levels on turtle grass flowering. Turtle grass exhibits plasticity in reproduction as a response to nutrient availability, whereby under low porewater nutrient conditions, resources are diverted to sexual reproduction rather than somatic growth. Anthropogenic nutrient loading, particularly in the form of groundwater

nutrient delivery, could decrease turtle grass flowering and potentially reduce genetic diversity of this species. This work could be extended through future studies focusing on the influence of stressors associated with coastal nutrient runoff into the water column such as epiphyte accumulation and light limitation on seagrass flowering, including investigations that focus on the existence of nutrient thresholds that are linked to flowering frequency.

5. Acknowledgements

We thank N. Fowler and P. Munguia for help with experimental design and data analysis and Z. Darnell and K. Jackson for assistance in the field and laboratory. T. Connelly provided valuable comments to early versions of the manuscript. This study was funded by the Estuarine Reserves Division, Office of Ocean and Coastal Resource Management, National Ocean Service, National Oceanic and Atmospheric Administration (Grant number NA11NOS4200082), an E.J. Lund Marine Science Research Fellowship and a Scholar Award from P.E.O. International.

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Tables

Table 1. Results of linear mixed models comparing shoots from unenriched and enriched turtle grass (*Thalassia testudinum*) plots in Lower Laguna Madre, Texas collected at the beginning of the experiment in March, prior to fertilization. Values are means ± S.E. None of the measured parameters were significantly different ($P > 0.05$) between unenriched and enriched plots.

	Unenriched	**Enriched**	***P***
Area of Longest Leaf (cm^2)	6.6 ± 2.4	6.7 ± 2.2	0.62
Leaf %C	35.8 ± 0.2	35.8 ± 0.2	0.84
Leaf %N	2.6 ± 0.0	2.6 ± 0.0	0.63
Leaf %P	0.2 ± 0.0	0.2 ± 0.0	0.62
Leaf molar C:N	16.5 ± 0.2	16.3 ± 0.3	0.91
Leaf molar C:P	556.7 ± 21.9	539.5 ± 26.2	0.69
Leaf molar N:P	33.2 ± 1.2	32.7 ± 1.2	0.66

533 Table 2. Results of linear mixed models comparing shoots from unenriched (unmanipulated) and
534 control (empty bag) turtle grass (*Thalassia testudinum*) plots in Lower Laguna Madre, Texas
535 collected at the end of the experiment in May. Values are means ± S.E. None of the measured
536 parameters were significantly different ($P > 0.05$) between unenriched (unmanipulated) and
537 control (empty bag) plots.

538

	Unenriched	**Control**	***P***
Porewater NH_4^+ (μM)	203.7 ± 34.7	170.9 ± 39.1	0.16
Proportion of Reproductive Shoots	0.2 ± 0.1	0.2 ± 0.0	0.68
Area of Longest Leaf (cm^2)	11.9 ± 0.9	12.0 ± 0.7	0.40
Aboveground Dry Wt. (g)	0.1 ± 0.0	0.1 ± 0.0	0.39
Leaf %C	37.0 ± 0.5	36.2 ± 0.4	0.55
Leaf %N	2.4 ± 0.1	2.2 ± 0.1	0.06
Leaf %P	0.2 ± 0.0	0.2 ± 0.0	0.89
Leaf molar C:N	18.4 ± 0.5	19.5 ± 1.0	0.14
Leaf molar C:P	499.4 ± 45.4	547.0 ± 57.1	0.60
Leaf molar N:P	27.1 ± 2.3	27.8 ± 2.2	0.23

539

Table 3. Results of linear mixed models comparing elemental composition of shoots from unenriched and enriched turtle grass (*Thalassia testudinum*) plots in in Lower Laguna Madre, Texas in May, at the conclusion of the experiment. Values are means ± S.E.

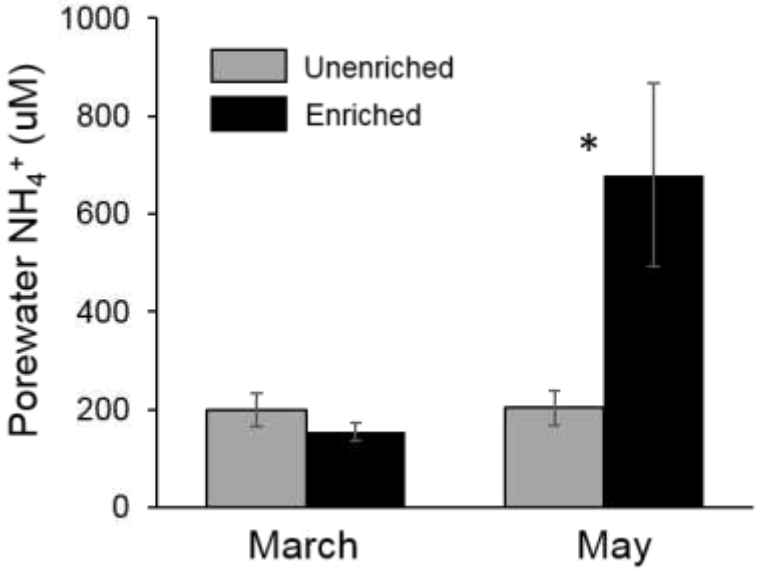
	Unenriched	**Enriched**	***P***
Leaf %C	36.84 ± 0.33	37.78 ± 0.17	0.01
Leaf %N	2.45 ± 0.05	2.90 ± 0.05	< 0.0001
Leaf %P	0.18 ± 0.01	0.18 ± 0.01	0.84
Leaf molar C:N	17.88 ± 0.39	15.41 ± 0.29	< 0.0001
Leaf molar C:P	565.81 ± 24.46	575.85 ± 21.45	0.74
Leaf molar N:P	30.40 ± 1.13	37.28 ± 1.20	< 0.0001

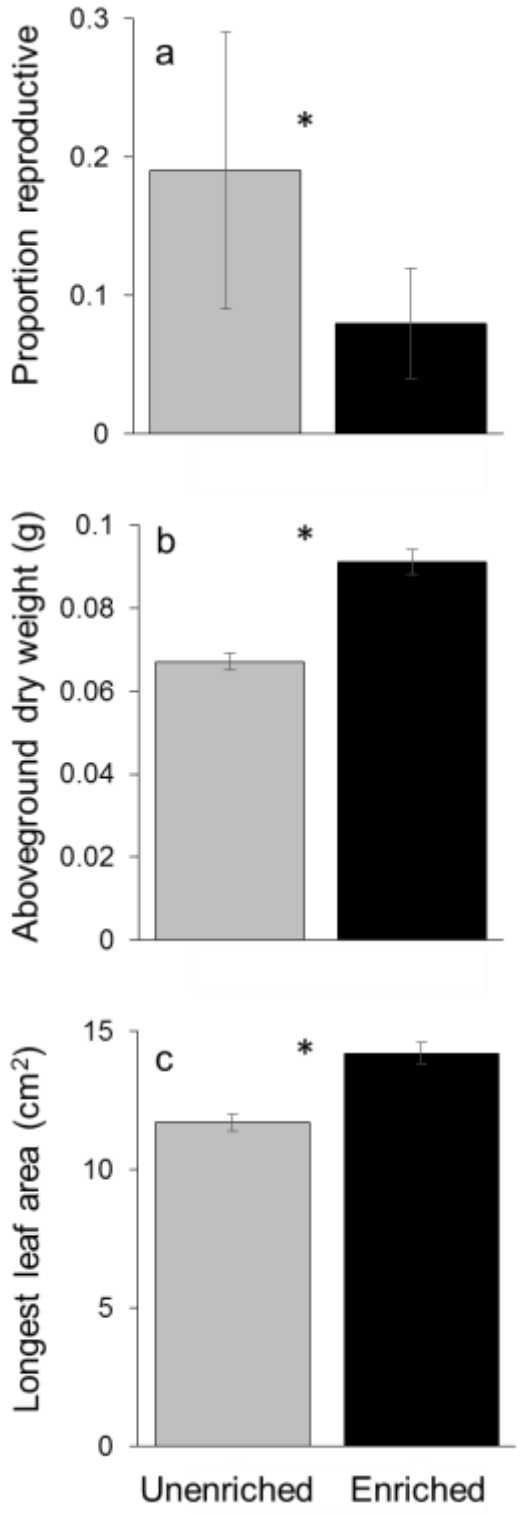
Figure Legends

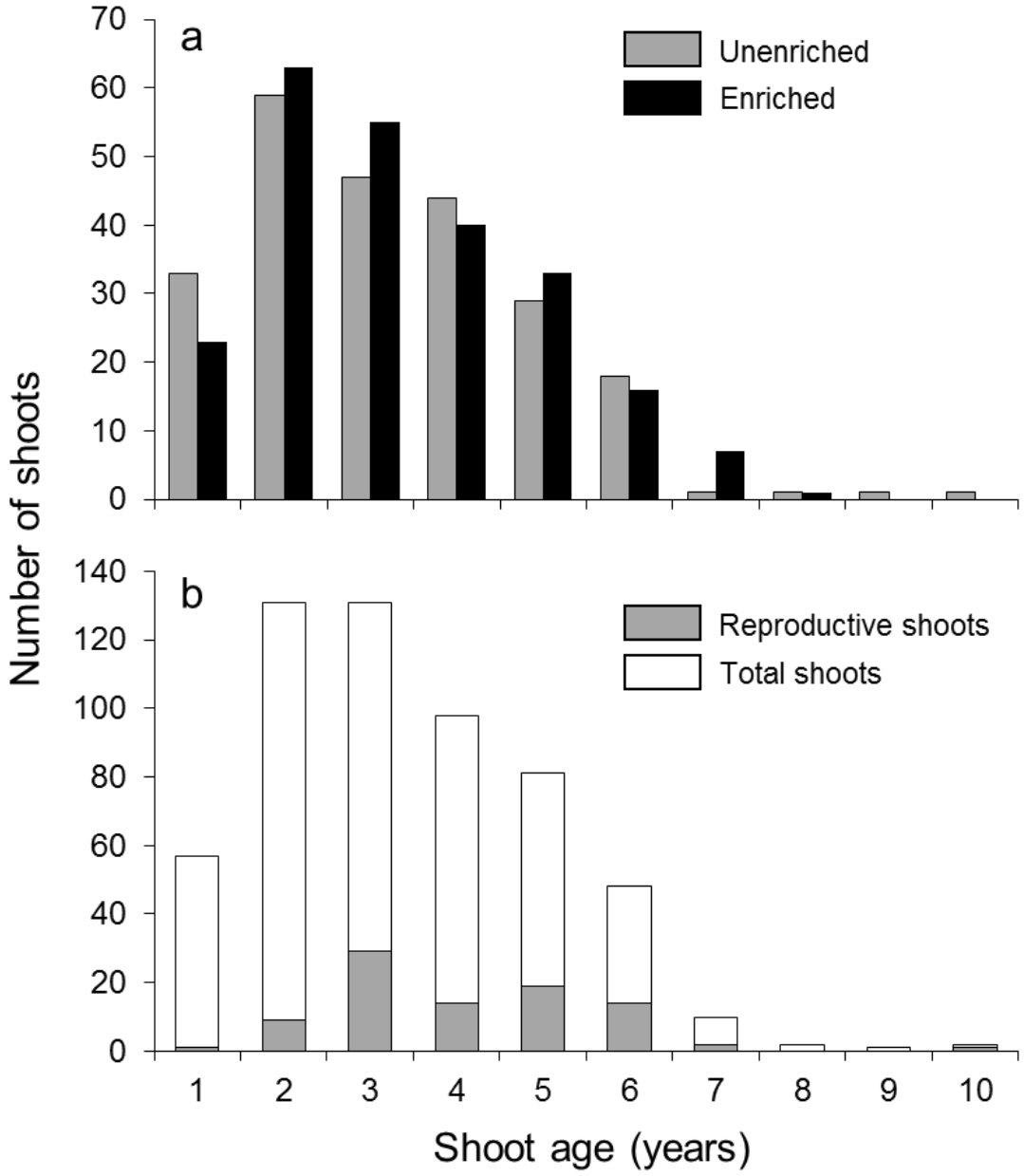
Fig. 1 Mean ± S.E. porewater NH_4^+ concentrations in plots unenriched and enriched with nitrogen where turtle grass (*Thalassia testudinum*) was collected at the beginning (March) and end (May) of the experiment. * denote significant differences

Fig. 2 Mean ± S.E. a) proportion of reproductive shoots, b) aboveground dry weight and c) area of longest leaf of turtle grass from plots unenriched and enriched with nitrogen at the end of the experiment in May. * denote significant differences

Fig. 3 Number of turtle grass shoots of each age a) from plots unenriched and enriched with nitrogen in May, and b) represented as the number of reproductive shoots and total number of combined unenriched and enriched shoots in May.


1000
800
600
400
200
0
Porewater NH_4^+ (uM)
Unenriched
Enriched
*
March
May


Proportion reproductive
0.3
0.2
0.1
0
a
*
Aboveground dry weight (g)
0.1
0.08
0.06
0.04
0.02
0
b
*
Longest leaf area (cm²)
15
10
5
0
c
*
Unenriched
Enriched


a
Unenriched
Enriched
70
60
50
40
30
20
10
0
b
Reproductive shoots
Total shoots
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Number of shoots
Shoot age (years)