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Interactive effects of hyposalinity and nitrate loading on growth, physiology, and nitrogen status of the seagrass, *Halodule wrightii*

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The study goal was to examine the interactive physiological effects of two freshwater inflow stressors, nitrate pulses coupled with salinity decrease, on the seagrass *Halodule wrightii*. A microcosm experiment was designed to approximate an observed freshwater inflow event. Over a 13-day period plants were subjected to three sequential salinity drops (S35→ S23→ S15→ S5) with nitrate-nitrogen added simultaneously at 0, 30 or 60 μ*M*. For comparisons, the Control was no salinity change and no nitrate added denoted by S35/No N. Measurements of *H. wrightii* shoot production, photosynthesis, respiration, quantum efficiency, %N, C:N ratios and δ^{15}N values which were made after each salinity drop revealed differing effects of low versus high N levels under S35 compared to reduced salinity. Compared to the Control at the experimental endpoint, leaf net photosynthesis: respiration (P:R) ratio decreased 3-fold for hyposalinity + High N addition (S5/High N) largely due to increased respiration. Leaf %N increased and C:N ratio decreased concomitantly with both stressors, with S5/High N having the highest %N and lowest C:N ratio. While the magnitude of the effect was related to the amount of added N at S35, there were different effects of Low versus High N at low salinity (S5). The trends of P:R ratio, leaf %N and C:N ratio are consistent with increased respiration, uptake of added N, and depletion of carbon reserves. However, δ^{15}N suggested that added NO3- was taken up by leaves at S35, but not at S5. The increased %N at S5 may be due to translocation of amino acid N from rhizomes-roots to leaves. Metabolic networks were hypothesized to be regulated differently at 30 versus 60 μ*M* NO3- under conditions of hyposalinity. These findings add to the growing evidence that simultaneous stressors typical of substantial freshwater inflow events, hyposalinity and nitrate loading, could adversely affect *H. wrightii*.

KEYWORDS

***Halodule wrightii*, hyposalinity, interactive stressors, nitrate enrichment, seagrass, stable nitrogen isotope ecology**

1 Introduction

Worldwide loss of seagrass has been well-documented (Orth et al., 2006; Waycott et al., 2009) with causes ranging from global climate change (increase in sea temperature, sea level, and frequency/intensity of storms), to regional changes in water quality and quantity and coastal development. Coastal freshwater input (FWI) can have significant effects on coastal ecosystems due to the quantity and quality of the FWI. Factors that influence FWI include land use/land cover, size of the watershed and the frequency and magnitude of rainfall events in the watershed. Coastal FWI is changing due to alterations of coastal watersheds and climate change (Ross and Randhir, 2022). With climate change, it is likely that coastal FWI events will be more intense and more frequent (Knutson, 2015; Kowalski et al., 2023). With coastal FWI comes depressed salinity, elevated water column N (inorganic and organic N) and light-attenuating turbidity (Kowalski et al., 2018; Mallin et al., 1999) which can lead to potential negative impacts on seagrass. Separately, hyposalinity and elevated N levels can, directly and indirectly, stress submerged aquatic vegetation. Although FWI events can include both hyposalinity and N loading, rarely has the combined effect of these two stressors on marine plants been examined.

1.1 Effects of salinity change on seagrass

Effects of salinity change on seagrass have been studied for years since salinity affects distribution of seagrass species (Kowalski et al., 2018; Lirman and Cropper, 2003; Biber, 2022), seagrass productivity (Kowalski et al., 2023), physiology (Koch et al., 2007; Kongrueang et al., 2018; Kowalski et al., 2024) and ultrastructure (Ferreira et al., 2017). Seagrass studies on hypersalinity are more numerous than studies on hyposalinity (478 *vs* 95 references, respectively) based on a search using BioAbstract, Science Direct and JSTOR.

Studies have documented the negative effects of hyposalinity on *Cymodocea nodosa*, *Z. notlii* and *Posidonia oceanica* (Invers et al., 2004; Fernández-Torquemada and Sánchez-Lizaso, 2011).

Hyposalinity was shown to lower photosynthetic rate, leaf chlorophyll concentrations, and quantum efficiency, while increasing respiration rate in various seagrass species (McMillan and Moseley, 1967; McMahon, 1968; Lirman and Cropper, 2003; Shafer et al., 2011; Fernández-Torquemada and Sánchez-Lizaso, 2011; Lamit and Tanaka, 2021; Gavin and Durako, 2014; Kowalski et al., 2023). Kongrueang et al. (2018) found that both hypo- and hypersaline conditions reduced the photosynthetic efficiency of the seagrass *Enhalus acoroides* seedlings. Osmotic stress due to hyposalinity can cause reduced growth and mortality resulting in substantial seagrass loss (Preen et al., 1995; Campbell and McKenzie, 2004; Griffin and Durako, 2012; DeYoe and Kowalski, 2014). A microcosm hyposalinity study for *Halodule wrightii* linked the physiological effects to cation depletion and notable shifts in the abundances of free amino acids, especially decreased proline and increased asparagine, among others, reflective of metabolic reprogramming (Kowalski et al., 2024). Plants exhibit broadly shared metabolic re-programming of carbon and amino acid metabolism in response to stressors (see Wang et al., 2018; Oddy et al., 2020; Alvarez et al., 2022 for reviews). For seagrasses, Udy and Dennison (1997) observed unique species-specific growth and biomass responses to N + P nutrient additions, yet there were similar underlying metabolic responses with respect to total amino acid levels, concentrations of two N-rich amino acids (N storage), and $\delta^{15}N$ of the leaves.

1.2 Effects of nitrogen loading on seagrass

Anthropogenic nitrogen (N) loading to estuaries has been seen to be problematic for more than 40 years (Jaworski, 1981; Nixon, 1990; Nixon et al., 2001; Bricker et al., 2008; Gao et al., 2016). FWI delivers N and suspended sediment that may degrade water quality with adverse effects on seagrass.

Prior to cultural eutrophication, occasional pulses of N-laden stormwater have been found to be beneficial for estuaries (Nixon, 1990). Nitrogen loading is influenced by watershed size, watershed land use/land cover characteristics and precipitation regime. The impact of N loading depends upon the nitrogen form (nitrate, ammonia, organic), amplitude of load, volume of the receiving water body and residence time. Microalgae, macroalgae, emergent plants and seagrasses are positioned to capture available N (Nielsen et al., 2004). Hauxwell and Valiela (2004) outlined a sequence of plant responses as increased N loading occurs. N-replete algae (phytoplankton and seaweeds) grow faster than submerged aquatic vegetation (SAV) (Reifel et al., 2009) with an increase in algal standing stocks (Lapointe and Matzie, 1996; Han et al., 2016; Shaw et al., 2018) that may cause SAV decline due to light limitation (Burkholder et al., 2007), but other indirect effects are possible. Generally, the scenarios discussed above reflect a continual delivery of N-replete input to coasts such as municipal/industrial wastewater and agricultural flows to estuaries. With storm events, N-laden stormwater inflow to estuaries occurs in a pulsed manner (McCorquodale et al., 2009; Kowalski et al., 2018; DeYoe et al., 2023) which has received less attention.

Climate change has increased the frequency, duration, and intensity of tropical cyclones (Webster et al., 2005; Knutson, 2015). This subsequently has/will increase FWI to coastal waters like the Lower Laguna Madre (LLM) of Texas through direct precipitation input and storm runoff (Kowalski et al., 2018). FWI can be delivered in a consistent baseline and/or pulsed fashion (Kowalski et al., 2018). DeYoe et al. (2023) stated that the LLM has a watershed to estuary area of 10:1 and a flushing time of 284 days, or 1.28 times per year. This residence time is potentially long enough that FWI of significant magnitude and concomitant N input could notably affect primary producers in the LLM (DeYoe et al., 2023).

Kowalski et al. (2023) examined incremental hyposalinity effects alone (35→23→15→5) on *H. wrightii* leaves on leaf growth, quantum efficiency, photosynthesis, and leaf respiration in microcosms. They found impaired quantum efficiency, leaf growth, photosynthesis, and increased respiration rates with

hyposaline conditions. In this study, the addition of water column N (as NO3-) was included to simulate the impacts of storm events (hyposalinity + N loading) on leaf growth and metabolism. N enrichment will increase N content for N-limited plants with physiological responses being species-specific (Lee et al., 2007; Touchette, 2007). The form of N is important as assimilation of NO3- is energetically more costly compared to ammonia and organic N (Touchette and Burkholder, 2000). The assimilation of inorganic N requires use of C skeletons to facilitate the transport and transfer of the N (Villazán et al., 2015) which may deplete plant carbon reserves (Touchette and Burkholder, 2000). For the seagrass *Zostera marina*, water-column NO3- enrichment with light reduction, or water-column NO3-enrichment with elevated temperature, caused lower shoot survival compared to single stressor treatments (Burkholder and Gilbert, 2013). Likewise, high ammonium levels paired with reduced light were hypothesized to impair *H. wrightii* (Kaldy et al., 2004).

Growth of N-limited seagrass can be stimulated by N influx (Irlandi et al., 2004; Govers et al., 2014; Mutchler and Hoffman, 2017; Hirst and Jenkins, 2017) resulting in increased seagrass leaf chlorophyll concentration and stimulation of photosynthesis (Lee and Dunton, 1999; Nielsen et al., 2004; Fourqurean et al., 2007). However, when Burkholder et al. (1994) subjected *H. wrightii*, *Ruppia maritima*, and *Z. marina* to 5 and 10 μ*M* NO3-, they found leaf growth in *Z. marina* declined while *H. wrightii* and *R. maritima* growth was stimulated. It is unclear if the effect on *Z. marina* was related to NO3- "toxicity" or a pH increase leading to CO2 limitation that accompanied the nitrate addition to tanks (Kaldy et al., 2022). Ammonium enrichment of the water column caused mortality in the seagrass *Z. noltii* when photosynthetic rates were not sufficient to supply enough carbon skeletons needed to assimilate nitrogen (Brun et al., 2002). Ammonium at levels as low as 25 μ*M* were found to be toxic to *Z. marina* (van Katwijk et al., 1997). The reduced form of N prevalent in anoxic sediments can be stimulatory to a point, but excessive NH4+ levels may be accompanied by high levels of toxic sulfide in anoxic sediments (Lee and Dunton, 2000). Stable N isotope measures ($\delta^{15}N$) are useful ecological tools because they integrate ecological processes and can be used as nutrient tracers (Lepoint et al., 2004). Dillon and Chanton (2008) used stable isotope values to assess the incorporation of $\delta^{15}N$ in tissues of macroalgae and seagrass. Under the influence of increased water column NO3- *H. wrightii* should become isotopically enriched relative to the Controls and incorporate ^{15}N from the water column N (as NO3-).

1.3 Interactive effects of hyposalinity and nitrogen loading

The negative impacts of environmental stressors can be exacerbated when they occur in combinations, however, not many studies have examined the interactive effects between hyposalinity and elevated water column N (Kahn and Durako, 2006; Stockbridge et al., 2020; Ostrowski et al., 2021). van Katwijk et al. (1999) found that *Z. marina* could tolerate high N levels (ammonium) at low salinity but not at high salinities. Jiang et al. (2013) explored interactions among two stressors, nutrients and salinity, for *Thalassa hemprichii*. "Pre-shading" plants before applying the stressors exacerbated the negative effects of hyposalinity and NO3- enrichment on leaf growth. The combination of pre-shading and reduced salinity induced non-structural carbohydrate (NSC) translocation from below-ground to above-ground parts and activated stress-related enzymes. Beck et al. (2024) found that seagrass declines were "somewhat associated" with increasing water temperatures and decreasing salinities and suggested that natural resource managers consider the reduced seagrass resilience due to these factors.

Two meta-studies concluded the need for more knowledge of how combinations of stressors interact and their types of interactions under realistic conditions (Stockbridge et al., 2020; Ostrowski et al., 2021). With climate change, increased frequency, intensity and duration of storms are predicted (Knutson, 2015). Amplified FWI events could place seagrass at risk, particularly in low FWI estuaries (Beck et al., 2024) like the LLM of Texas, so an understanding of the combined impacts of hyposalinity and elevated nitrogen levels on seagrasses is needed. This study addressed how the combination of hyposalinity and elevated NO3- levels could impair *H. wrightii* physiology (leaf growth, net photosynthesis, quantum efficiency, and leaf respiration), and indicators of N metabolism (leaf %N content, molar C:N ratios and stable isotope $\delta^{15}N$ incorporation) under conditions approximating an observed FWI event.

2 Materials and methods

2.1 Study site and plant collection

Halodule wrightii Ascherson (shoal grass) was selected for this study because it is a widespread euryhaline tropical seagrass. Plants for this microcosm study were collected as 10-cm diameter cores in July 2016 from a shallow water (50 cm depth) site in uniformly dense (5,000-8,000 shoots m^{-2}) monotypic *H. wrightii* beds in the Lower Laguna Madre of Texas (26.14850, -97.18200). Water column temperature (28.7°C), salinity (37.4), pH (7.97) and dissolved oxygen (DO) (5.25 mg l^{-1}) were measured with a calibrated Hydrolab Quanta multiprobe at the time of collection. Seawater from the site (chlorophyll <1 μg l^{-1}; and NO3- <5 μ*M*), collected the same day as seagrass, was used to fill 18 aquarium tanks at The University of Texas Rio Grande Valley, Edinburg, Texas. Plants with associated sediment were potted (10 cm diameter, 13 cm deep) in the field, covered with a tarp and transported within 6 hours of collection to be placed into 38 l aquaria (51 x 28 x 30.5 cm) filled with 27 l of seawater to a depth of 20 cm.

2.2 Culture

Seven pots were placed in each of 18 aquaria in a temperature-controlled chamber (Norlake Scientific, Hudson, WI). Aquarium pumps (flow rate of 200 L hr^{-1}) were used for circulation. Light was provided with low heat T5 high output fluorescent white lights on a 14:10 light-dark cycle (320 µmol photons m^{-2} s^{-1}) (Dunton, 1994). Plants were acclimated for 7 days at S35 before initiation of the experiment. Water temperature, salinity, pH, and dissolved oxygen were recorded every other day. Salinity was adjusted by adding deionized water. Tank water temperature was 25°C (± 2°C). Pots were placed randomly in aquaria and relocated daily to reduce the likelihood of a potential bias effect of location within an aquarium. Aquarium walls were cleaned daily to reduce development of periphyton. Aquaria were assigned a salinity and nitrogen treatment (3 replicates per treatment) using a random number generator in Microsoft Excel.

2.3 Treatments

2.3.1 Pulsed salinity decrease

Of the 18 tanks used in the experiment, nine were maintained at S35 throughout the experiment (Figure 1). In the other nine tanks (designated S5 endpoint), salinity was reduced sequentially (Drops 1–3 respectively) from S35 to S23 to S15 then S5. Each intermediate hyposalinity was maintained for several days to allow time for metabolic and physiological adjustments specifically, 4 days at S23, 6 days at S15, and 3 days at S5. For the initial 7-day acclimation period and each subsequent intermediate salinity period, the first

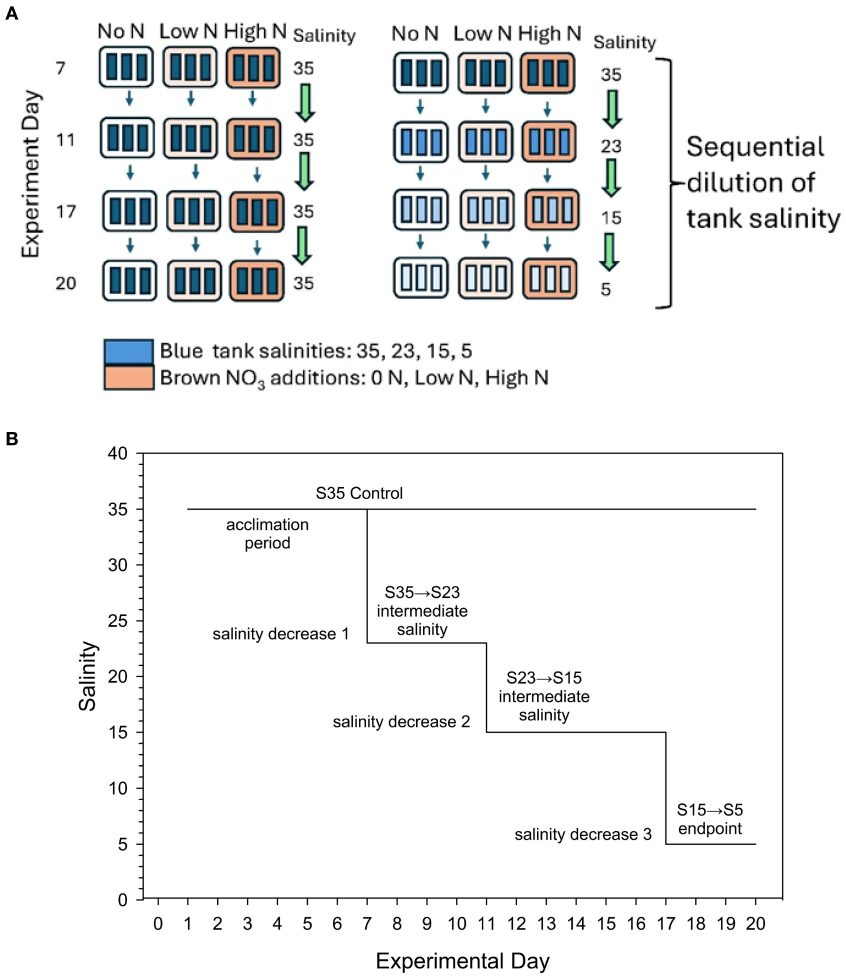


FIGURE 1
Experimental design and salinity drop schedule for microcosms with Control (S35/No N), hyposalinity (S5) and added nitrate (as KNO3) at two levels (30 µ*M* and 60 µ*M* NO3-). **(A)** Experimental aquaria setup showing salinity and nitrogen treatments. **(B)** Salinity reduction scheme during the 20-day experiment. Besides the 9 salinity reduction aquaria, there were 9 aquaria that were maintained at a salinity of 35 (Control) for the duration of the experiment. Following Drop 1, salinity reduction aquaria remained at reduced salinity for 4 days, then 6 days following Drop 2 and 3 days following Drop 3. Nitrate spikes were added to appropriate aquaria after each salinity change.

and last days (salinity drops on days 7, 11, and 17) were approximately half of the day at the higher salinity and half of the day at the lower salinity. When averaged over time, salinity change rates approximated the maximum mean salinity change rates of the water column following the Hurricane Alex freshet of 2010 (Kowalski et al., 2018).

2.3.2 Nitrogen additions

Aquaria were also randomly assigned as a N Control (No N, no N added), Low N treatment (30 μ*M* NO3-), or High N treatment (60 μ*M* NO3-) (Figure 1). Levels and forms of nitrogen in storm runoff are highly variable (Jani et al., 2020). But nitrate is typically a large component of N in FWI (Jani et al., 2020) immediately available to plants. Added levels of NO3- N were based on observations of 108 and 14 μ*M* NO3- levels in a storm runoff channel and a nearby seagrass bed, respectively (DeYoe et al., 2023). Most LLM seagrass beds have ~ 1 μ*M* or less of NO3- outside of storm events, and average N:P ratios of ~23 (Cuddy and Dunton, 2023). All treatment combinations were done in triplicate aquaria. To maintain N concentrations that occur during extended stormwater runoff events (Mallin et al., 1999), the Low and High N aquaria were amended with KNO3 to the treatment concentrations the same day that salinity reductions occurred. Water samples were collected from each aquarium for water column nitrite-nitrate analysis before and after N additions. The source KNO3 used for the N additions had a $\delta^{15}N$ value of -2.75‰. The cadmium reduction method (Parsons et al., 1984) was used for nitrite-nitrate assays.

2.4 Leaf carbon and nitrogen

At the end of the experiment (Day 20), 100 mg wet weight of leaf tissue was removed from each aquarium, rinsed in distilled water, dried at 70°C for 2 days, ground to a fine powder and weighed to the nearest μg for ^{15}N, percent C and N analysis, and calculation of molar C and N ratios. Leaf samples were analyzed for N isotope ratios and leaf C and N content at the University of California Davis Stable Isotope Facility with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were not acidified to remove inorganic carbon since acidification can alter $\delta^{15}N$ signatures (Ng et al., 2007). Final $\delta^{15}N$ values are expressed relative to international air standards for N.

2.5 Shoot production

The leaf-puncture method of Kowalski et al. (2001) was used to measure shoot production rates. Pots were randomly selected from each replicate treatment aquarium, and 20 shoots were pierced with a 28-gauge hypodermic needle (<0.5 mm diameter) just above the basal meristem on the first day of the 13-day (not counting the 7-d acclimation period) experiment. A rubber ring was placed at the base of pierced shoots so they could be located at the end of the experiment. On the last day of the experiment, marked shoots were detached from the rhizome and shoots were rinsed in deionized water and leaf segments were examined under a dissecting microscope to confirm that only pierced shoots and attendant leaves were collected. Leaves were gently scraped to remove epiphytes, measured for leaf length from the hole where shoots were pierced then new growth excised and dried at 70°C for 2 days, then weighed to the nearest 0.001 mg. Mean shoot growth was calculated as the mass of dried leaves divided by the number of shoots divided by time (days) for growth. All leaves produced per shoot were dried and weighed and shoot production rates expressed as mg dw $shoot^{-1}$ d^{-1}.

2.6 Photosynthesis

Photosynthesis measurements (O2 evolution) were made in the laboratory using Fire Sting (PyroScience, Inc., Germany) optical dissolved oxygen (DO) fixed mini-sensor (3 mm diameter) probes interfaced with a 4-channel meter connected to a computer. Shoots were selected randomly from pots. As photosynthetic rates along a leaf vary from tip to sheath (Enríquez and Borowitzka, 2010), only the youngest emerged leaf per shoot that was long enough to obtain a 4 cm length section was used. For each measurement, one leaf from each of three shoots were placed between two clear 1 cm x 4 cm plastic mesh sheets to hold the leaves side-by-side and perpendicular to the light source to eliminate shading (Shafer et al., 2011).

To minimize accumulation of O2 in the leaf, a 10-minute lag allowed lacunal O2 concentrations to equilibrate with the surrounding water column (Herzka and Dunton, 1997). For photosynthesis measurements, O2 levels were reduced to 50% saturation or less by bubbling with N2 gas. Circulation was achieved by including a small magnetic stirrer in the reaction vessel which was placed on a magnetic stir plate. DO measurements for photosynthesis were made under saturating irradiance (ca. 320 μmol photons m^{-2} s^{-1}). Three 100 W cool, white light LED bulbs were positioned perpendicular to the leaves in the reaction chambers. Dissolved oxygen measurements were logged once every five minutes for 30 minutes. Net photosynthetic rates were calculated by the difference in DO concentrations (final-initial) multiplied by chamber volume, corrected by subtracting the value of a blank, and divided by incubation time and leaf mass and expressed as μmol O2 mg^{-1} dw leaf hr^{-1}.

2.7 Photosynthesis quantum efficiency

A portable pulse amplitude-modulated (PAM) fluorometer (Junior-PAM, Walz, Germany) was used to measure photosynthetic quantum efficiency (Fv/Fm; QE) on leaf sections. Leaf sections were situated 1 mm from the tip of the 1 mm diameter light pipe, held at a constant 60° angle to the leaf (see Gavin and Durako, 2014 for details). The leaf sections were submersed in treatment water of a given salinity and then covered with an opaque black fabric cloth for 15 minutes of dark acclimation prior to measurement.

2.8 Respiration

Respiration measurements were done in the same manner as photosynthesis measurements using the same leaf segments employed for photosynthesis measurements. The leaves were held in the dark for 15 minutes to draw-down photosynthetic metabolites (respiratory substrates) and diminish light-enhanced dark respiration (Griffin and Turnbull, 2012). Dissolved oxygen measurements were logged once every minute for 20 minutes. Leaf segments were rinsed in de-ionized water, dried at 70°C for 2 days, and weighed to the nearest 0.001 mg. Rates for respiration were calculated as with photosynthesis. The net photosynthetic rate (P) was divided by the respiration rate (R) to calculate the P:R ratio.

2.9 Statistics

The data were collected from eighteen different tanks at several follow-up time points; therefore, the measurements of the outcome variables shoot production, photosynthesis, quantum efficiency, leaf respiration rates, photosynthesis to respiration ratios, percent N, molar C:N ratios and isotopic signatures were expected to be correlated within each tank. Each aquarium was treated as a statistical replicate (N = 3) with final endpoint salinity of 5, compared to a Control salinity at 35. Generalized Estimating Equations (GEE) models were used to analyze the outcomes variables accounting for the within tank responses' correlation by incorporating predefined "working" correlation structures to estimate the regression parameters and their uncertainty (Zeger and Liang, 1986). GEE are known as marginal or population-averaged models with parameter estimates representing the average difference between subjects (Zeger and Liang, 1986). The principle interest of the analysis was to test the difference in mean outcomes between the salinity and N treatment and one Control group in each of the salinity drop's time intervals, therefore GEE models were fitted using the main effect of the salinity treatment and salinity drop and the interaction term between salinity and nitrogen treatment and salinity drop. The QIC statistic was used to select the best model's "working" correlation structure (Hilbe, 2007). Tukey-Kramer test was used for *post-hoc* multiple pairwise comparisons of mean outcome between groups. All statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., 2023). All statistical tests were two-sided and were performed using a significance (α) level of 0.05.

3 Results

Table 1 summarizes the GEE findings for all three salinity drops. Measures of N dynamics (%N, C:N molar ratios and $\delta^{15}N$ signatures) were more sensitive to interactions compared to measures of metabolic performance (quantum efficiency, net photosynthesis, respiration and net photosynthesis/respiration ratios (P:R ratio)). There were significant differences within and between group comparisons for salinity ($p < 0.001$) and N additions ($p < 0.001$) for photosynthesis, leaf respiration, quantum efficiency, $\delta^{15}N$, %N and C:N molar ratios (Table 1).

TABLE 1 Estimated interaction effects between nitrogen levels and salinity levels using Generalized Estimating Equations (GEE) models to analyze *Halodule wrightii* variations in net photosynthesis, respiration rates, net photosynthesis to leaf respiration ratios, quantum efficiency, leaf percent nitrogen, leaf C:N molar ratios and leaf δ15N, as functions of salinity drops (S23, S15 and S5) and KNO3 additions at 30 μ*M* (Low N) and 60 μ*M*, (High N), compared to S35 control with no N additions (No N).

Effect	Drop #1		Drop #2		Drop #3	
	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value
Outcome variable: leaf net photosynthetic rates						
High N: S35 *vs.* Salinity trt	30.50 (2.47)	**<0.0001**	-20.31 (3.19)	**<0.0001**	-14.58 (3.25)	**0.0010**
Low N: S35 *vs.* Salinity trt	71.60 (6.24)	**<0.0001**	-43.26 (0.78)	**<0.0001**	-41.01 (6.09)	**<0.0001**
No N: S35 *vs.* Salinity trt	6.25 (2.36)	0.4201	-24.64 (1.33)	**<0.0001**	-11.35 (2.64)	**0.0023**
S35: High N *vs.* Low N	-39.66 (6.13)	**<0.0001**	-16.48 (1.91)	**<0.0001**	-25.41 (1.55)	**<0.0001**
S35: High N *vs.* No N	28.63 (1.87)	**<0.0001**	-11.47 (1.96)	**<0.0001**	-10.68 (2.14)	**<0.0001**
S35: Low N *vs.* No N	68.29 (6.36)	**<0.0001**	5.01 (0.72)	**<0.0001**	14.74 (2.15)	**<0.0001**

(Continued)

TABLE 1 Continued

Effect	Drop #1		Drop #2		Drop #3	
	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value
Outcome variable: leaf net photosynthetic rates						
Salinity trt: High N *vs.* Low N	1.44 (2.72)	1	-39.43 (2.68)	**<0.0001**	-51.84 (6.6)	**<0.0001**
Salinity trt: High N *vs.* No N	4.38 (2.87)	0.9886	-15.80 (2.85)	**<0.0001**	-7.44 (3.59)	0.8317
Salinity trt: Low N *vs.* No N	2.94 (2.02)	0.9933	23.63 (1.36)	**<0.0001**	44.39 (6.21)	**<0.0001**
Outcome variable: leaf respiration rates						
High N: S35 *vs.* Salinity trt	-3.82 (2.23)	0.9650	-27.02 (3.51)	**<0.0001**	-68.83 (9.22)	**<0.0001**
Low N: S35 *vs.* Salinity trt	-22.9 (7.17)	0.1206	-28.23 (5.59)	**<0.0001**	-157.61 (8.29)	**<0.0001**
No N: S35 *vs.* Salinity trt	23.65 (2.6)	**<0.0001**	-90.07 (2.44)	**<0.0001**	-55.75 (6.56)	**<0.0001**
S35: High N *vs.* Low N	-14.46 (5.15)	0.3059	-26.6 (5.53)	**0.0002**	-3.6 (3.07)	0.9995
S35: High N *vs.* No N	3.48 (2.41)	0.9940	46.12 (3.45)	**<0.0001**	44.63 (2.74)	**<0.0001**
S35: Low N *vs.* No N	17.94 (5.52)	0.1037	72.72 (4.43)	**<0.0001**	48.23 (1.58)	**<0.0001**
Salinity trt: High N *vs.* Low N	-33.54 (5.46)	**<0.0000**	-27.81 (3.6)	**<0.0001**	-92.38 (12.01)	**<0.0001**
Salinity trt: High N *vs.* No N	30.95 (2.43)	**<0.0001**	-16.93 (2.53)	**<0.0001**	57.71 (10.98)	**<0.0001**
Salinity trt: Low N *vs.* No N	64.49 (5.25)	**<0.0001**	10.88 (4.2)	0.4586	150.1 (10.45)	**<0.0001**
Outcome variable: net photosynthesis:respiration ratio						
High N: S35 *vs.* Salinity trt	0.27 (0.05)	**<0.0001**	-0.04 (0.02)	0.9793	0.21 (0.04)	**<0.0001**
Low N: S35 *vs.* Salinity trt	0.51 (0.12)	**0.0028**	-0.18 (0.04)	**0.0002**	0.26 (0.04)	**<0.0001**
No N: S35 *vs.* Salinity trt	-0.46 (0.05)	**<0.0001**	0.39 (0.01)	**<0.000**	0.34 (0.03)	**<0.0001**
S35: High N *vs.* Low N	-0.18 (0.12)	0.9868	-0.08 (0.03)	0.4560	-0.11 (0.04)	0.4444
S35: High N *vs.* No N	0.27 (0.01)	**<0.0001**	-0.48 (0.02)	**<0.0001**	-0.44 (0.05)	**<0.0001**
S35: Low N *vs.* No N	0.45 (0.11)	**0.0101**	-0.4 (0.03)	**<0.0001**	-0.33 (0.04)	**<0.0001**
Salinity trt: High N *vs.* Low N	0.07 (0.06)	0.9999	-0.23 (0.03)	**<0.0001**	-0.06 (0.03)	0.9283
Salinity trt: High N *vs.* No N	-0.46 (0.07)	**<0.0001**	-0.05 (0.02)	0.1226	-0.31 (0.02)	**<0.0001**

(Continued)

TABLE 1 Continued

Effect	Drop #1		Drop #2		Drop #3	
	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value
Outcome variable: net photosynthesis:respiration ratio						
Salinity trt: Low N *vs.* No N	-0.52 (0.06)	**<0.000**	0.18 (0.03)	**<0.000**	-0.25 (0.03)	**<0.0001**
Outcome variable: quantum efficiency						
High N: S35 *vs.* Salinity trt	-0.01 (0.002)	0.2035	-0.020 (0.01)	**0.0020**	0.07 (0.01)	**<0.0001**
Low N: S35 *vs.* Salinity trt	-0.004 (0.01)	1	0.002 (0.01)	1	0.002 (0.001)	0.9843
No N: S35 *vs.* Salinity trt	-0.01 (0.01)	0.8785	0.010 (0.004)	**0.0242**	-0.03 (0.004)	**<0.0001**
S35: High N *vs.* Low N	0.01 (0.004)	0.8888	0.020 (0.003)	**<0.0001**	0.02 (0.01)	**0.0045**
S35: High N *vs.* No N	0.02 (0.002)	**<0.0001**	0.030 (0.01)	**<0.0001**	0.03 (0.01)	**<0.0001**
S35: Low N *vs.* No N	0.01 (0.004)	**0.0307**	0.020 (0.01)	0.0679	0.01 (0.002)	**<0.0001**
Salinity trt: High N *vs.* Low N	0.01 (0.003)	0.3627	-0.004 (0.01)	1	-0.05 (0.001)	**<0.0001**
Salinity trt: High N *vs.* No N	0.02 (0.01)	0.1467	0.002 (0.004)	1	-0.06 (0.003)	**<0.0001**
Salinity trt: Low N *vs.* No N	0.01 (0.01)	0.9984	0.010 (0.01)	1	-0.02 (0.004)	**0.0005**
Outcome variable: leaf %N						
High N: S35 *vs.* Salinity trt	0.29 (0.02)	**<0.0001**	-0.18 (0.002)	**<0.0001**	-0.55 (0.02)	**<0.0001**
Low N: S35 *vs.* Salinity trt	0.56 (0.01)	**<0.0001**	0.02 (0.01)	0.9451	-0.06 (0.02)	0.3817
No N: S35 *vs.* Salinity trt	0.03 (0.01)	0.1239	0.53 (0.02)	**<0.0001**	-0.49 (0.01)	**<0.0001**
S35: High N *vs.* Low N	0.03 (0.01)	0.1338	0.04 (0.01)	0.2543	0.1 (0.02)	**<0.0001**
S35: High N *vs.* No N	0.20 (0.01)	**<0.0001**	0.54 (0.01)	**<0.0001**	0.28 (0.02)	**<0.0001**
S35: Low N *vs.* No N	0.16 (0.01)	**<0.000**	0.50 (0.02)	**<0.0001**	0.18 (0.01)	**<0.0001**
Salinity trt: High N *vs.* Low N	0.3 (0.02)	**<0.0001**	0.24 (0.004)	**<0.0001**	0.59 (0.02)	**<0.0001**
Salinity trt: High N *vs.* No N	-0.13 (0.02)	**<0.0001**	0.19 (0.01)	**<0.0001**	0.34 (0.01)	**<0.0001**
Salinity trt: Low N *vs.* No N	-0.43 (0.01)	**<0.0001**	-0.05 (0.01)	**0.0082**	-0.25 (0.02)	**<0.0001**
Outcome variable: leaf C to N ratio						
High N: S35 *vs.* Salinity trt	-2.09 (0.06)	**<0.0001**	0.82 (0.08)	**<0.0001**	2.89 (0.13)	**<0.0001**

(Continued)

TABLE 1 Continued

Effect	Drop #1		Drop #2		Drop #3	
	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value	Mean difference (SE)	Tukey-Kramer adjusted p-value
Outcome variable: leaf C to N ratio						
Low N: S35 *vs.* Salinity trt	-6.42 (0.08)	**<0.0001**	-0.92 (0.05)	**<0.0001**	-0.73 (0.04)	**<0.0001**
No N: S35 *vs.* Salinity trt	-0.16 (0.08)	0.8320	5.00 (0.11)	**<0.0001**	4.32 (0.04)	**<0.0001**
S35: High N *vs.* Low N	-0.41 (0.02)	**<0.0001**	-0.14 (0.07)	0.8937	-1.1 (0.1)	**<0.0001**
S35: High N *vs.* No N	-1.72 (0.04)	**<0.0001**	-5.79 (0.09)	**<0.0001**	-3.6 (0.11)	**<0.0001**
S35: Low N *vs.* No N	-1.31 (0.04)	**<0.0001**	-5.65 (0.06)	**<0.0001**	2.55 (0.04)	**<0.0001**
Salinity trt: High N *vs.* Low N	-4.74 (0.1)	**<0.0001**	-1.88 (0.05)	**<0.0001**	-4.71 (0.09)	**<0.0001**
Salinity trt: High N *vs.* No N	0.21 (0.09)	0.6216	-1.61 (0.09)	**<0.0001**	-2.16 (0.08)	**<0.0001**
Salinity trt: Low N *vs.* No N	4.95 (0.1)	**<0.0001**	0.27 (0.1)	0.3679	-2.5 (0.04)	**<0.0001**
Outcome variable: leaf δ15N						
High N: S35 *vs.* Salinity trt	-0.58 (0.15)	**0.0103**	0.54 (0.14)	**0.0194**	-0.57 (0.14)	**0.0104**
Low N: S35 *vs.* Salinity trt	-0.56 (0.23)	0.5843	0.21 (0.06)	0.0905	-0.53 (0.09)	**<0.0001**
No N: S35 *vs.* Salinity trt	1.65 (0.1)	**<0.0001**	-0.48 (0.12)	**0.0126**	0.12 (0.14)	1
S35: High N *vs.* Low N	-1.09 (0.13)	**<0.0001**	-0.13 (0.04)	0.1843	-0.27 (0.15)	0.9360
S35: High N *vs.* No N	-1.04 (0.12)	**<0.0001**	-0.1 (0.12)	1	-0.58 (0.16)	**0.0227**
S35: Low N *vs.* No N	0.05 (0.13)	1	0.03 (0.12)	1	-0.32 (0.08)	**0.0030**
Salinity trt: High N *vs.* Low N	-1.08 (0.24)	**0.0010**	-0.46 (0.15)	0.1806	-0.23 (0.09)	0.3960
Salinity trt: High N *vs.* No N	1.19 (0.13)	**<0.0001**	-0.16 (0.14)	0.9997	0.11 (0.12)	1
Salinity trt: Low N *vs.* No N	2.27 (0.22)	**<0.0001**	0.3 (0.07)	**0.0033**	0.34 (0.15)	0.6854

Units for each parameter: net photosynthesis and respiration rates, μmol O2 g-1 dw leaf hr-1; photosynthesis to respiration ratios (unitless); quantum efficiency, Fv/Fm; Leaf nitrogen as % nitrogen; C:N ratios, carbon and nitrogen moles; δ15N as ‰.
Bold face values indicate statistically significant differences.

3.1 Shoot production

Effects of hyposalinity or N treatments on shoot production rates were not notable, except for a 20% decrease for the S5/No N treatment compared to S35/No N (Figure 2; P = 0.05). N added treatments had no effect on mean shoot production at S35 but at S5 added N did stimulate shoot production, i.e., S5/Low N was 45% greater while S5/High N was 28% greater compared to S5/No N. Pairwise differences in shoot production were nearly different by salinity without added N (P = 0.05) and significantly by Low N treatment at S5 (P = 0.004; P = 0.051 for High N) and salinity-N interaction (P = 0.037).

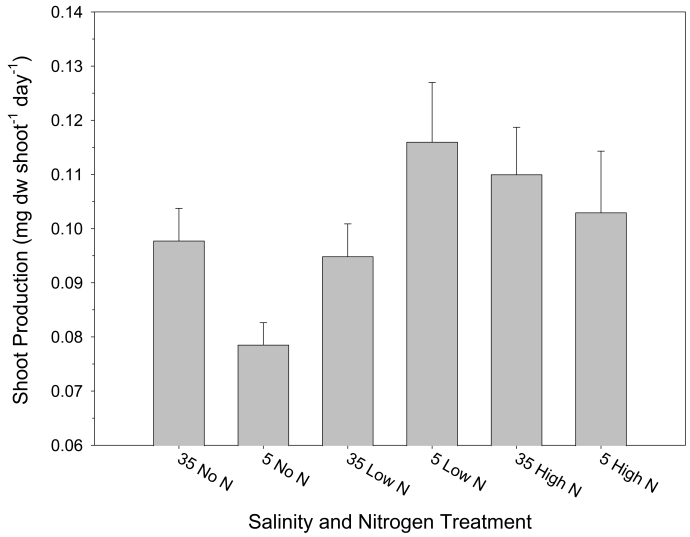


FIGURE 2
Halodule wrightii mean shoot production (mg dw shoot d^{-1}) (± SEM) at S35 (control) and S5. Nitrogen treatments (as KNO3) were 30 µ*M* (Low N) and 60 µ*M* (High N). N = 6 to 11.

3.2 Net photosynthesis and quantum efficiency

Seagrass maximum net photosynthetic rates were significantly affected by all treatments (Figure 3; Table 1). The photosynthetic rate at S5/Low N was about 2-fold higher than S35/No N Control and was 60% higher than the S5/No N treatment. The Low N treatment at S35 was only 23% greater compared to the S35/No N Control. Net photosynthetic rates for High N treatments were ~15% lower than their respective No N Controls for both salinities. Thus, there were stimulatory versus inhibitory effects of the low and high N additions, respectively, on net photosynthesis. The effects of salinity treatment and N additions were highly significant ($P < 0.001$, $P = 0.002$, respectively), as was the salinity-nitrogen interaction (Table 1).

Quantum efficiency increased significantly with added N at S35 ($P < 0.001$) but did just the opposite at S5 i.e., small increases (~ 4%) with N addition at S35 were in contrast to S5 where N addition reduced QE by 2% at Low N and 8% at High N (Figure 3). The S5/High N plants had the lowest QE value (0.7) which was highly significant ($P < 0.0001$). QE for hyposalinity alone (S5/No N) was slightly higher (4%) than the S35/No N Control value.

3.2 Respiration

Leaf respiration rates were significantly stimulated by both hyposalinity and N treatments in an interactive manner (Figure 3, Table 1). Separately, the influence of salinity and N treatments were highly significant ($P < 0.001$), as was salinity-N treatment interaction ($P = 0.009$) (Table 1). Rates were consistently greater at low salinity (S5) compared to S35 at either N treatment. The combination of hyposalinity and either N addition treatment increased respiration rate more than either hyposalinity or N addition alone. In comparison to the S35/No N Control, leaf respiration increased 2-fold for hyposalinity or 1.5 to 1.8-fold for N treatments individually, while at S5, the N treatments further increased respiration 1.5 to 2.3-fold. Respiration from the combined S5/+N addition treatments were 3 to 5-fold greater compared to the S35/No N Control. Although there was an increasing respiration trend with added N at S35, at S5/High N respiration was lower than the S5/Low N value, inconsistent with the trend at S35 (Figure 3).

3.3 Net photosynthesis to respiration ratio (P:R)

Except for S35/No N Control (P:R ~1.2), the mean P:R ratios for all treatments were less than 0.7 at the end of the experiment (Figure 3). Hyposalinity alone decreased the ratio to 0.7. Added N decreased the P:R ratios in a concentration dependent manner at both salinities, but the relative magnitude of decrease from the added N was much greater at S35 than at S5, with final ratios of 0.6 and 0.4, respectively. Despite the high individual rates for P and R separately in the S5/Low N treatment, its P:R ratio was similar to that of the other two S5 treatments.

3.4 *Halodule* leaf %N, C:N ratios and $\delta^{15}N$

In comparison to S35/No N (Control), all treatments had lower C:N molar ratios and higher %N levels (Figure 4). Regardless of salinity, High N resulted in greater changes than Low N for C:N and %N. The leaf % N content was 1.8% in the S35/No N but was higher in all other treatments by ~10 to 50% relatively. At S35, the %N increase trended proportionally with the added N concentration,

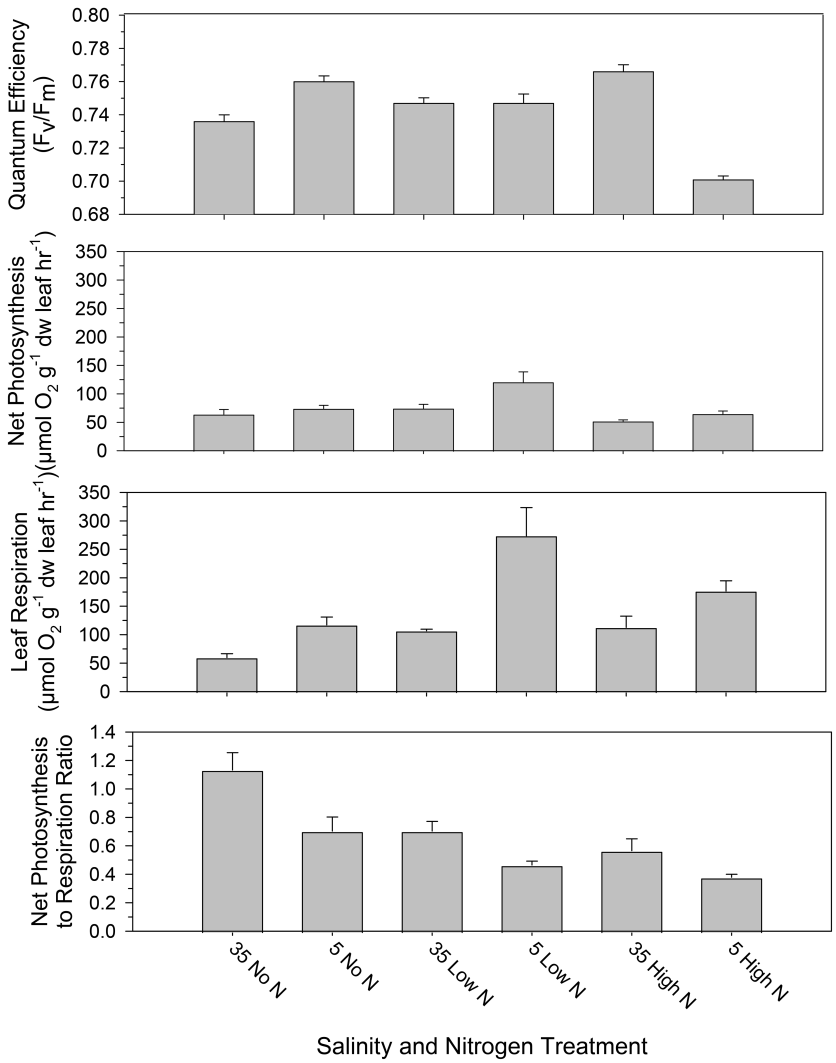


FIGURE 3
Halodule wrightii mean values (± SEM) for quantum efficiency, net photosynthesis and respiration rates, and net photosynthesis to respiration ratios only at experimental endpoint (salinity Drop 3). Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M* (High N) compared to control with no N additions (No N). Where there is no error bar, error is smaller than error symbol. N = 3 for control and treatments. Graphs for each parameter at all drops (intermediate salinities) are in Supplementary Material.

consistent with assimilation of supplemented N. Nitrogen addition at S5 did not show a consistent trend: S5/No N was ~2.3%, S5/Low N was lower at ~2.0% with the highest %N for S5/High N plants (~2.8%). Salinity treatment, N treatment, and salinity-N treatment interaction were all significantly different ($P < 0.001$) (Table 1).

Leaf C:N ratios decreased from 22.5 in Control to as low as 16.5 for all treatments, concomitant with the increases in %N (Figure 4; Table 1). The greatest N-induced changes occurred for the High N treatments at both salinities (-16% and -27% at S35 and S5, respectively). Salinity treatment, N treatment, and salinity-N treatment interaction were all significant ($P < 0.001$) (Table 1). The S5 Low N values for C:N and %N did not fit the N-induced trends in these parameters observed at S35, but these results reflect the combined impact of added N with hyposalinity.

The $\delta^{15}N$ value at S35 was reduced for Low (+2.70‰) and High N (+2.44‰) treatments compared to No N (+3.01‰) consistent with uptake of added NO3- ($P = 0.009$) (Figure 4, Table 1). The $\delta^{15}N$ value of KNO3 used for N additions was -2.75‰. In contrast to the S35 results, and seemingly counter to the measured increase of % N and decrease of C:N at S5 hyposalinity, the $\delta^{15}N$ value for S5/No N (+2.89‰) was lower than the values for S5/Low N (+3.23‰) or S5/High N (+3.00‰) treatments suggesting no uptake of the added N. Overall, $\delta^{15}N$ differences for salinity alone were significant ($P = 0.004$) and significant for salinity-N interaction ($P = 0.014$), but not for N alone (Table 1). N-induced differences relative to the No N treatments were altered by hyposalinity, supporting an interactive effect of hyposalinity and N addition.

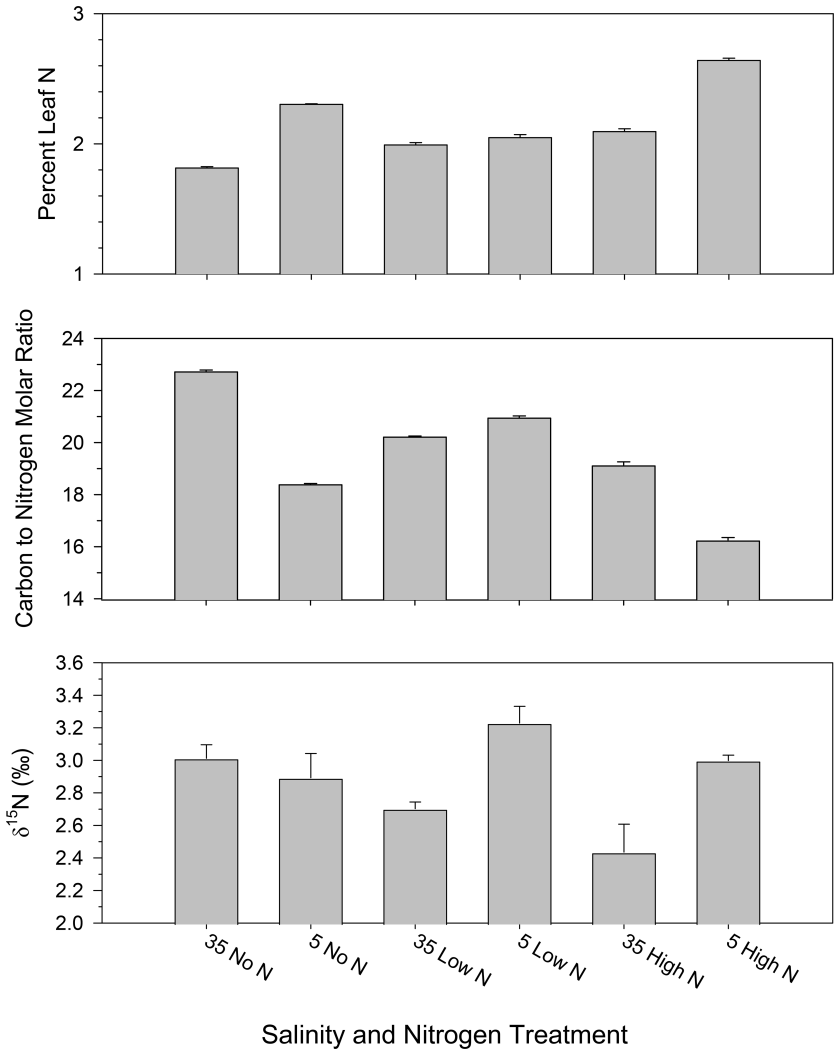


FIGURE 4
Halodule wrightii mean values (± SEM) for leaf percent nitrogen, leaf C:N molar ratios, and leaf $\delta^{15}N$ (‰). Only experimental endpoint Drop 3 data are shown. Plants were subjected to N levels (as NO3-) at 30 μ*M* (Low N) and 60 μ*M* (High N) compared to control No N addition (No N). Where there is no error bar, error is smaller than error symbol. N = 3 for controls and treatments. Graphs for each parameter at all drops are in Supplementary Material.

4 Discussion

This experiment compared the individual and combined effects of hyposalinity and two levels of NO3- on *H. wrightii* shoot production, physiological parameters and indicators of N status/metabolism. Leaf growth integrated the response to the stressors whereas the physiological and nitrogen indicators revealed shorter term underlying metabolic responses.

Mean shoot growth decreased ~20% with hyposalinity treatment alone (P = 0.05) in this short-term experiment. Nitrate additions had no significant effects at normal salinity (S35), but NO3- addition combined with hyposalinity resulted in greater leaf production compared to S5/No N (P = 0.04), suggesting compensation of hyposalinity-induced growth inhibition. For the physiological parameters, all hyposalinity and nitrogen treatments maintained or increased rates of net photosynthesis and respiration, but the P:R ratio decreased significantly for both treatments compared to the Control (Figure 3) and there were additive negative effects of the two stressors that were N concentration dependent.

However, for 5 of the 7 physiological parameters at S5, the Low N values were either lower or higher, respectively, than No N and High N treatments. The P:R ratio decreased up to 3-fold, primarily due to additively increased respiration from both hyposalinity and NO3- addition. Leaf %N increased and C:N ratio decreased concomitantly with applied stressors. The greatest change was observed for the combination of hyposalinity and high N addition. These measures are consistent with uptake of added N, increased respiration and depletion of carbon. However, there were disparate effects of Low versus High N at S5. At S35, the magnitudes of the observed effects trended with the amount of added N and $\delta^{15}N$ decreased consistent with uptake of added NO3-. However, at

S5, the lack of $\delta^{15}N$ significance suggested instead that added NO3- was not taken up by the leaves, despite increased %N and increased shoot production by N addition. This increased leaf N in the absence of isotopic evidence of nitrate uptake at S5 is best interpreted as combined stressor-induced alteration of C- and/or N-metabolisms for which there is evidence in multiple species and stressors (Bian et al., 2020; Alvarez et al., 2022; Kowalski et al., 2024). We hypothesize these alterations involve translocation of stored N (most likely as amino acids) from rhizomes-roots to the leaves (Jiang et al., 2013; Tegeder and Masclaux-Daubresse, 2018; Vega et al., 2019; Wang et al., 2021; Yang et al., 2023) as this could increase leaf N in the absence of external N uptake. The interactive effects of hyposalinity and NO3- are consistent with integrated C- and N-metabolism (Bian et al., 2020; Oddy et al., 2020; Carillo and Rouphael, 2022) and further suggest different metabolic networks at 30 versus 60 μ*M* NO3- under hyposalinity, evidenced by the differing effects of Low *vs* High N additions. These interpretations contribute testable hypotheses for future – omics studies needed to resolve plant responses to interacting FWI stressors.

Regardless of treatment (hyposalinity and/or N addition), there was shoot production (Figure 2). Even though stressors had mixed effects on photosynthesis while increasing respiration, there were sufficient carbon reserves (probably from rhizomes) available in the plants (Wang et al., 2021; Jiang et al., 2013) to accommodate shoot growth for the duration of the experiment. Shoot production values from this study are similar to those from an earlier *H. wrightii* study for the LLM of Texas (Kowalski et al., 2009). Perhaps this experiment was too short (20 days) to see negative effects on shoot production, but it is notable that NO3- addition ameliorated the growth inhibition at hyposalinity without impacting growth at normal salinity. This suggests that leaves were not N-limited at S5 during the experiment since they did grow without apparent uptake and assimilation of external N as suggested by the leaf $\delta^{15}N$ data.

4.1 Physiological parameters

The physiological parameters revealed significant and patterned responses to individual and combined stressors (Figure 3). Hyposalinity alone increased net photosynthesis (~15%, non-significant), QE (~4%), and doubled respiration. Low and High N additions had, respectively, stimulatory and inhibitory (or less positive) effects on net photosynthetic rates at both salinities. The differences were not significant at S35, but notable and significant for S5/Low N. The N additions also increased QE (+4% for High N) at S35 but significantly decreased QE at S5 (-8% at High N). *R. maritima* exhibited an almost immediate decrease in photosynthesis after salinity decreased from 35 to 10, however, it could adapt osmotically over a period of 1–2 days (Murphy et al., 2003). Although our experiment was relatively short (13 days at various hyposalinities), measurements after each salinity drop showed progressive changes in physiological parameters (Supplementary Figures S1-S7) which may represent adjustments in C metabolism as discussed below. The decline in QE may serve as a means to avoid damage to the photosynthetic apparatus when plants are under stress (Niyogi, 2000). Kongrueang et al. (2018) found that both hypo- and hypersaline conditions reduced the QE of the seagrass *Enhalus acoroides* seedlings.

Respiration at S35 nearly doubled with N addition regardless of N concentration, but at S5, Low N addition respiration rates were 2.3-fold greater than S5 No N and ~4.6-fold greater than the S35 Control. In contrast, High N had a less positive effect on respiration at S5 (only ~1.5-fold that of the S5 No N).

The ratio of net photosynthesis to respiration (P:R) revealed the balance between the two major energy production/expenditure processes involved in carbon metabolism. Except for S35/No N Control,*bH. wrightii* P:R ratios for all treatments were 0.7 or less, and significantly less than the Control (1.1) (Figure 3). This implies that the Control (S35/No N) produced more reserve carbon potentially for use in growth and reproduction than plants from the other treatments. The sharp increase in leaf respiration rates in this study was likely due to the high simultaneous energy costs of hyposalinity (Shafer et al., 2011; Kowalski et al., 2024) and assimilation of N by leaves (Touchette and Burkholder, 2000). This is notable since it implies that under conditions of hyposalinity and excessive nitrogen loading there is potential to decrease the resiliency of *H. wrightii* due to reduced capacity to produce reserve carbon (non-structural carbon reserves) for growth or reproduction (Jiang et al., 2013). Further studies of longer duration are needed to explore potential linkage to seagrass resiliency.

The contrasting effects of Low *vs* High N additions and the different salinities on photosynthesis, QE and respiration, positive at low and negative (or less positive) at high N concentrations, were unexpected and suggest metabolic reprogramming (Xu and Fu, 2022) from the interactive stressors. Low N had a greater stimulatory effect on photosynthetic rate than did High N at either salinity and QE increased or decreased, respectively, by N additions at S35 or S5. Similarly, Low N had a greater stimulatory effect on respiration than did High N only at S5. Increased respiration appears to be a response of *H. wrightii* to both hyposalinity and N addition separately, but their interaction is complex. The range of average leaf respiration rates in this study was comparable to field measurements of *H. wrightii* respiration by Dunton (1996).

The lesser effects on photosynthesis and respiration at High N compared to Low N (at both salinities) might result from dysregulation of N metabolism (Tegeder and Masclaux-Daubresse, 2018; Vega et al., 2019; Shilpha et al., 2023) and subsequent disruption of C metabolism (Coschigano et al., 1998; Britto and Kronzucker, 2002; van der Heide et al., 2008; Tsuno et al., 2011; Villazán et al., 2015; Wang et al., 2018; Carillo and Rouphael, 2022) due to organic acid depletion (Paul and Pellny, 2003). NO3- itself can serve as a regulatory molecule triggering gene expression changes that divert carbon, including decreased starch synthesis and increased production of organic acids and amino acids in plants (Wang et al., 2018; Carillo and Rouphael, 2022). Different metabolic networks at 30 versus 60 μ*M* NO3- under conditions of hyposalinity would explain the observed disparate effects of low *vs* high N additions. Future studies with proteomics and metabolomics

techniques could reveal how these metabolic networks shift (Xu and Fu, 2022). As noted by Jiang et al. (2013), seagrass with diminished non-structural carbohydrate (NSC) reserves may be especially susceptible to stressors where the plant response relies on NSC as a source of osmoticum and carbon skeletons for ammonium assimilation. NSC reserves decreased for the combination of reduced salinity and NO3- enrichment in *T. hemprichii* (Jiang et al., 2013).

NSC reserves likely supported a large part of the increased respiration for energy production in this study. Benjamin et al. (1999) found that the rhizome diameter of *Halophila ovalis* decreased when plants were grown at a reduced salinity (10), suggesting that the plants were expending rhizome carbon (NSC) under hyposaline conditions. Murphy et al. (2003) found reduced starch reserves and increased soluble sugars under hyposalinity in *R. maritima*.

4.2 Effects of stressors on N status and metabolism indicators

The N status measures showed increased leaf N content and decreased C:N ratios for both N treatments and hyposalinity (Figure 4). Hyposalinity alone (S5/No N) resulted in the %N value being 27% higher compared to Control, indicating that stress can increase leaf N in the absence of added N. The magnitude of the % N change was related to the amount of N added with one exception (S5 Low N), and the effect was greatest when hyposalinity was combined with the highest level of N (46% *vs* Control). Only at S35 did the observed δ^{15}N changes of *H. wrightii* leaves provide evidence of N incorporation from added NO3- in the water column. The leaf δ^{15}N values decreased as expected (i.e. became more depleted by up to 0.5‰, a decrease of 19%) for the uptake of external N with a δ^{15}N value of -2.75‰. The initial S35 Control δ^{15}N value of ~+3 was typical for *H. wrightii* (Cuddy and Dunton, 2023).

The nitrate-induced changes in δ^{15}N seen at S35 were not seen at S5 however. The S5 mean leaf δ^{15}N values did not change significantly compared to the original S35 Control, and despite 17 - 46% increases in %N with 0 - 60 μ*M* N additions (Figure 4, Table 1). For hyposalinity stress alone, %N increased in the absence of added N, and when N was added at S5, there was no new N assimilation apparent based on δ^{15}N values, despite changes in physiological parameters. This implies another source for the increased N, such as translocation of N from rhizomes-roots to the leaves, as seen for eight seagrass species by Marbà et al. (2002). If this is the case, then alteration of N-metabolism, and reallocation is suggested (Kaldy, 2011; Marbà et al., 2002) to explain increased leaf N in the absence of new N assimilation (Hemminga et al., 1991; Wang et al., 2018; Yang et al., 2023). In a parallel study, Kowalski et al. (2024) found that the same S5 hyposalinity treatment depleted the leaves of ions (including K^+, Cl^-, Ca^{+2}), osmoticum, and, by inference, C skeletons. These ions are all regulatory and could potentially signal altered C metabolism. The large pool of free proline (1 N atom) amino acids was replaced by a new large pool of asparagine (2 N atoms) (Kowalski et al., 2024), a mechanism that would reduce osmoticum and make more C available for respiration while conserving N levels.

Energy from NSC (Jiang et al., 2013), and C skeletons from amino acids could be translocated from below-ground stores to shoots. The N-rich amino acids might retain their original δ^{15}N signature yet add to the N content of the leaf tissue so the leaf δ^{15}N value would not be greatly affected, assuming leaves and rhizomes-roots have nearly the same δ^{15}N signature. This scenario is consistent with our observations and suggests that NO3- uptake into leaves was either blocked (Sandoval-Gil et al., 2019) and/or affected by N secretion (Bian et al., 2020) of the absorbed NO3-. The latter explanation would only work if the N secretion process were highly selective for the lighter N isotope, and, importantly, if the N translocation into leaves were biased to favor the heavier N isotope (which is unlikely). We thus favor the interpretation that the added NO3- was not taken up into leaves. As we did not measure the N content of rhizomes-roots in this study, future work should examine this scenario.

Coordination of whole plant response is complex and, beyond the immediate scope of this work, likely involves additional metabolisms related to homeostasis of ions, pH and phosphorus (Ye et al., 2022). Phloem NO3- transport also has an important role in NO3- homeostasis (Tegeder and Masclaux-Daubresse, 2018) and plant growth regulation, and many NO3- transporters in rice and *Arabidopsis* have been characterized as to their contributions towards N allocation for growth and stress response (Sun et al., 2013; Wang et al., 2018; Ye et al., 2022; Carillo and Rouphael, 2022). These concepts are consistent with a large body of research on the regulation of nitrogen use efficiency (NUE) and impacts of stressors in model plant systems (Sun et al., 2013; Wang et al., 2018; Landrein et al., 2018; Carillo and Rouphael, 2022; Xu and Fu, 2022; Yang et al., 2023). It is reasonable to expect some conservation of these functions in the seagrasses.

The *H. wrightii* leaves used for all of the assays in this work were young (but at least 4 cm in length) to avoid complications with epiphyte fouling. By the end of the 3rd drop most remaining leaves represented new growth which would have developed under the environmental conditions of the applied stressors and probably drew N and C reserves from the root/rhizome (Marbà et al., 2002; Wang et al., 2018; Ota et al., 2020; Yang et al., 2023).

4.3 Changes during the salinity drop regime

The foregoing was based primarily on differences observed at the experimental endpoint i.e., after Drop 3. Since salinity was reduced in three steps (35→23→15→5) during the experiment, hyposalinity stress was presumably exacerbated over time. Supplementary Figures S1-S7 show details of measured physiological and N status parameters with each salinity drop. At Drop 1, δ^{15}N at S23 Low or High N were not lower than the No N. For the same time at S35, δ^{15}N values for only High N (δ^{15}N=1.85) were reduced compared to No N treatment (δ^{15}N=2.88). After Drop

2, there was ^{15}N uptake by S35/High N ($\delta^{15}N$ = 2.72) compared to the respective No N treatment ($\delta^{15}N$=2.82). It appears that ^{15}N uptake was initiated at the time of Drop 1 but only in the S35 High N treatments. At experimental endpoint (Drop 3), leaf $\delta^{15}N$ did indicate significant ^{15}N uptake at S35 Low and High N but not by Low or High N at S5. Added N combined with hyposalinity seemed to impact the $\delta^{15}N$ dynamics throughout the experiment.

4.4 Potential implications of combined stressor impacts

Gavin and Durako (2014) and Griffin and Durako (2012) found that under low salinity (5 and 10), *H. johnsonii* tolerated short-term reductions better than prolonged salinity reductions. Benjamin et al. (1999) found *H. ovalis* variably intolerant to prolonged hyposalinity. Long-term exposure to hyposalinity would cost plants stored carbon through diversion to respiration instead of being used for growth and reproduction. Seagrasses in general may be more vulnerable when rhizome carbon reserves are low, e.g. due to hyposalinity, nitrogen loading (Jiang et al., 2013), light-limited conditions (Kaldy et al., 2004; Jiang et al., 2013) or early in the growing season (Kowalski et al., 2009). DeYoe et al. (2023) suggested that seagrass decline in the LLM of Texas was related to the combined effects of hyposalinity and nitrogen loading.

5 Conclusions

The purpose of this study was to examine the combined effects of two common freshwater inflow stressors, hyposalinity and nitrogen loading, on the seagrass *H. wrightii*. The energy loss from combined hyposalinity and N stressors, increased leaf %N, and $\delta^{15}N$-indicated differences in uptake of external NO3- at the different salinities suggested interactive regulation of plant responses related to whole plant coordination of N- and C-metabolisms and shoot development. Different metabolic networks were presumably at work at 30 versus 60 μ*M* NO3- under conditions of hyposalinity, but not so at S35. Coordination of whole plant response is complex and there remains an incomplete understanding of N assimilation and reallocation during the simultaneous leaf growth and senescence that occurs in *H. wrightii* so it is not yet possible to predict seagrass bed or ecosystem level impacts from the physiological responses. Moreover, while added N appeared to relieve the growth impairment observed from hyposalinity, the magnitude and timing of multiple stressor exposures could lead to great variation in resulting impacts. Further studies are required to enable modeling of the potential impacts for seagrass management (Stockbridge et al., 2020; Ostrowski et al., 2021).

A major implication of this study is that simultaneous stressors, hyposalinity and N loading, typical of substantial FWI events, could potentially have detrimental effects on *H. wrightii* with respect to plant resilience. This phenomenon might apply to other seagrass species also. If so, coastal managers and seagrass conservation efforts should consider the impacts the combined effects of salinity regimes and nitrogen loading have on seagrass persistence.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JK: Writing – original draft, Writing – review & editing, Formal Analysis, Investigation, Methodology, Resources. HD: Writing – review & editing, Formal Analysis. KC: Funding acquisition, Resources, Writing – original draft, Writing – review & editing, Investigation, Methodology. KV: Formal Analysis, Writing – review & editing, Methodology.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2025.1712666/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Quantum efficiency mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

SUPPLEMENTARY FIGURE 2
Photosynthesis mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

SUPPLEMENTARY FIGURE 3
Leaf respiration mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

SUPPLEMENTARY FIGURE 4
Photosynthesis to respiration ratio mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

SUPPLEMENTARY FIGURE 5
Leaf percent nitrogen mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

SUPPLEMENTARY FIGURE 6
Leaf C:N molar ratio mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

SUPPLEMENTARY FIGURE 7
Leaf δ^{15}N (‰) mean values (± SEM) for *Halodule wrightii* from each of the three salinity drop and nitrogen treatment regimes (See Figure 1). Bars labeled by each treatment endpoint. Salinity values for drops 1–3 were, respectively, 23, 15 or 5. Plants were subjected to N levels (as KNO3) at 30 µ*M* (Low N) and 60 µ*M*, (High N) compared to control with no N additions (No N). Where no error bar is seen, error is smaller than error symbol. N = 3 for control and treatments.

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