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Meadow trophic status regulates the nitrogen filter function of tropical seagrasses in seasonally eutrophic coastal waters

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Abstract

The nutrient filter function is an important ecosystem service of seagrass meadows that mitigates the consequences of coastal eutrophication. In northeast Hainan in China, large seagrass areas were lost due to chronic eutrophication induced by untreated pond aquaculture effluents. However, in adjacent areas, seagrasses could survive due to seasonal exposure (i.e., not chronic) to eutrophication only. In a way, the conditions in these areas represent a transitional environment which allows investigating the effect of eutrophication on seagrass performance and their nitrogen uptake capacity. We tested how a 4-week in situ nutrient enrichment affected inorganic nitrogen uptake rates of a multispecies seagrass meadow in eutrophic and non-eutrophic seasons, in light and in darkness. All species maintained nitrogen uptake in the dark and preferred ammonium over nitrate. In the eutrophic season, the seagrass leaf biomass and growth were lower resulting in a lower nitrogen filter capacity. Among the species present, *Cymodocea rotundata* and *Cymodocea serrulata* covered 48% and 45%, respectively, of their daily nitrogen demand for leaf growth through leaf uptake from the water column, while it was only 30% for *Thalassia hemprichii*, the last remaining species in meadows degraded by eutrophication, as deduced from previous studies. It indicates that a multispecies seagrass meadow has a higher nitrogen filter capacity than a monospecific *T. hemprichii* meadow. By reducing seagrass diversity and, hence, the nitrogen filter function, eutrophication triggers a self-reinforcing process. Once the nitrogen filtering capacity of a seagrass meadow is exhausted, further eutrophication and seagrass loss are expected.

Seagrasses inhabit shallow coastal waters at the land-sea interface. They rank among the most productive ecosystems and have a multitude of ecosystem services (Nordlund et al. 2016). To sustain the high primary productivity, seagrasses assimilate nitrogen and other nutrients from the environment, thereby limiting the bioavailability for algae, improving water quality, and mitigating the risk of eutrophication (van der Heide et al. 2007). Low nutrient availability limits seagrass productivity (Powell et al. 1989; Agawin et al. 1996); however, nutrient supply exceeding the filtering capacity of a seagrass meadow leads to eutrophication, which is one of the major threats to seagrass ecosystems (Burkholder et al. 2007).

In eutrophic ecosystems, excess nutrients trigger the growth of algae (both phytoplankton and macroalgae), which, if not exported from the system, ultimately can lead to an increase in the organic matter concentration, anoxic sediment conditions, and a reduction of light availability to seagrass. Anthropogenic nitrogen input and the associated eutrophication have been related to biomass and biodiversity losses in seagrass meadows worldwide, especially in poorly flushed estuaries, where nutrient loads are high and frequent (Burkholder et al. 2007).

Among the consequences of eutrophication, nutrient enrichment and decreased light availability have been related to hamper seagrass performance (Herbeck et al. 2014). Several indicators for elevated nitrogen exposure have been identified in seagrasses, such as an initial increase of seagrass growth (Agawin et al. 1996; Terrados et al. 1999), elevated total nitrogen (TN) concentrations

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in tissues (Agawin et al. 1996), an altered morphology (Thomsen et al. 2020, 2022), or a reduction of carbohydrate reserves in belowground biomass (Invers et al. 2004). Conversely, low light availability reduces growth rates, induces the shedding of leaves, and reduces carbohydrate reserves in belowground biomass (*see* review by McMahon et al. 2013 and references therein). These changes may ultimately affect seagrass meadows' ecosystem functions and services, including their nitrogen sink function; however, the impact of eutrophication on this ecosystem service has yet received little attention.

Nitrogen assimilation is directly related to photosynthetic activity as the process requires energy and carbohydrates. In this sense, in some terrestrial plants and algae, prolonged light deprivation may impair nitrogen uptake (Delhon et al. 1995; Pereira et al. 2008). In contrast, some seagrass species can maintain stable nitrogen uptake rates in the darkness (Lee and Dunton 1999; Alexandre et al. 2015) by catabolizing its carbohydrate reserves (Alexandre et al. 2015).

In general, the preferred nitrogen sources are ammonium (NH_4^+) followed by nitrate (NO_3^-) through leaf uptake (Viana et al. 2019) and NH_4^+ through belowground uptake (Touchette and Burkholder 2000). During increased availability of inorganic nutrients in the water column, leaf nitrogen uptake gains in importance over belowground uptake (Iizumi and Hattori 1982). The preference of NH_4^+ over NO_3^- is energetically advantageous for the plants, as NO_3^- uptake involves an energy-demanding active transport system (Touchette and Burkholder 2000). At certain nitrogen concentrations, NO_3^- uptake is suppressed, while NH_4^+ uptake increases (Alexandre et al. 2010). As the physiology and morphology differ among species, different seagrass species may become winners or losers under the same changing scenarios.

In southern China, coastal waters are under high anthropogenic pressure, especially from aquaculture (Herbeck et al. 2013; Hu et al. 2021). In particular, in northeast (NE) Hainan, the eutrophication resulting from aquaculture effluent inputs has caused an 87% seagrass decline in the last decade (Thomsen et al. 2020), with varying risk depending on sites and seasons (Herbeck et al. 2013). Therefore, this area is well suited to study how anthropogenic eutrophication affects the performance of seagrass species and consequently their ecosystem services, such as the nitrogen filter function.

This study aimed to determine how different trophic states affect inorganic nitrogen uptake by leaves of seagrass species; as well as leaf biomass, growth rate and nitrogen (N) demand, related with the nitrogen filter function. For this purpose, we investigated an artificially fertilized plot and a control plot within a multispecies seagrass meadow on the coast of Hainan (China) in two different seasons (dry and wet). In each season, we quantified the leaf uptake rates of NH_4^+ and NO_3^- of the species present at that time under light and dark conditions, once the desired trophic status was reached. We further estimated leaf biomass and growth and N demand to finally discuss how these changes in plants' performance are related to seagrass meadows' nitrogen filtering capacity.

We hypothesized that (1) leaf NO_3^- uptake is the highest at low nitrogen conditions, while NH_4^+ uptake is the highest at high nitrogen concentrations. The cumulative (2) leaf inorganic nitrogen uptake is higher under fertilized conditions than under control conditions. Furthermore, (3) species with higher TN reserves have a higher nitrogen demand and uptake than species with low TN in the tissues.

Methods

Study area and site

The experimental site Yelin (19°31.3′N, 110°51.9′E) is located NE of the Wenchang–Wenjiao Estuary outlet at the NE coast of the Hainan Island China. Hainan's climate is characterized by a warm, sunny, and rainy season with regular typhoon impact from May to November and a cold, dry, and cloudy season from December to April. The annual rainfall ranges from 1500 to 2000 mm. The coastal zone of NE Hainan is covered by large areas (~ 4000 ha) of aquaculture ponds. In fact, the areal conversion of mangrove forests to aquaculture ponds accelerated in the mid-1970s and reached its current extent by 2000 (Herbeck et al. 2020). Consequently, seagrass species composition shifted from dense multispecies meadows to a sparse presence of *Thalassia hemprichii* and *Enhalus acoroides* (Thomsen et al. 2020). Overall, 87% of the seagrass biomass in NE Hainan was lost between 2009 and 2017 due to eutrophication caused by aquaculture effluents; at sites with direct discharge all seagrass was lost (Thomsen et al. 2020).

The experimental site is remotely affected by aquaculture ponds' effluents, but its waters are periodically eutrophied when typhoons and heavy rainfall wash out nutrient and organic matter-rich waters from ponds that fringe the Wenchang–Wenjiao Estuary (Herbeck et al. 2011). Comparatively high nitrogen stable isotope values ($\delta^{15}N$) of up to 9‰ in *T. hemprichii* leaves (Herbeck et al. 2014; Thomsen et al. 2020) and phytoplankton bioassays (Herbeck and Unger 2013) indicate periodical eutrophication from aquaculture effluent input.

The experimental seagrass meadow was at 0.9 m water depth. Six seagrass species grow in a 1.5 km^2 backreef area. *T. hemprichii* is the most abundant, followed by *Cymodocea serrulata*, *Cymodocea rotundata*, and *Syringodium isoetifolium*. Close to the coast, *Halodule uninervis* and *Halophila ovalis* grow intermixed with the other species (Herbeck et al. 2014; Thomsen et al. 2020).

Experimental design and fertilization procedure

We in *situ* fertilized an area of 2.5 m^2 of the seagrass meadow while keeping a plot of the same size with no fertilization during the dry and wet seasons (Fig. 1). The experimental areas were 120–150 m away from the shore. To secure independence between the seasons, the experimental areas in

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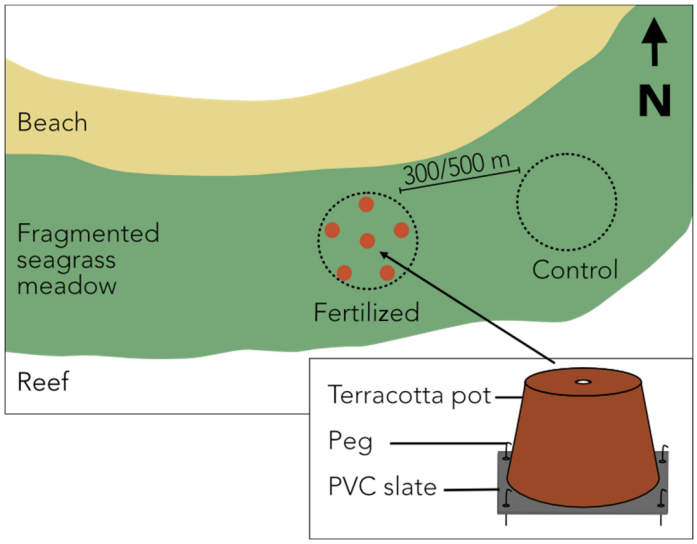


Fig. 1. Experimental set-up in the back-reef area in Yelin (northeast coast of Hainan). For fertilization, we filled six terracotta pots with slow-release fertilizer and installed them at the experimental site. The control and fertilized sites were 300 m apart in dry season and 500 m apart in the wet season. This figure is not true to scale.

the dry season were not the same as in the wet season. The control and the fertilized treatments in the seagrass meadow were 300 m apart in the dry season and 500 m in the wet season (Fig. 1).

For the water column fertilization, we sealed open-pored terracotta flowerpots at the top-opening using nonpermeable polyvinyl chloride (PVC) slates. The flowerpots were filled with 1 kg of commercial balanced (NPK = 15–15–15, N–P_2O_5–K_2O) compound fertilizer with ≥ 45% nutrients according to the Chinese standard GB 15063 2009. Of the 15% nitrogen in the fertilizer, approximately 40% is ammonium and 60% is nitrate. The filled and sealed flowerpots were placed upside-down in the seagrass meadow with the PVC slate facing the sediment and secured with pegs (Fig. 1). The fertilizer could diffuse through the open-pored terracotta into the water column. Five of these flowerpots were arranged in the experimental plot forming a pentagon shape of 2.5 m^2 with a 6th pot in the center. Weekly, the fertilizer residues and algae growing in the pots were removed, and the pots were refilled with 1 kg of fertilizer.

The fertilization lasted from 25 April 2017 to 22 May 2017 (27 d) in the dry season and from 21 September 2017 to 26 October 2017 (35 d) in the wet, typhoon season. Two weeks after the fertilization started in the wet season, the weather changed to excessive rain and wind until the end of the experiment.

At the end of the fertilization experiments, during seagrass collection for growth measurements (*see* "Seagrass leaf biomass, leaf growth, and nitrogen demand" section), additional 4–5 seagrass plants of each species present at each season were sampled within the experimental plots, next to the six quadrates per plot and stored on ice. These plants were transported to the laboratory and cleaned from epiphytes, divided into leaves, roots, and rhizomes. The newly grown 2 cm of the second youngest leaves from all seagrass plants of the same species were pooled, dried at 40°C until constant weight, homogenized with mortar and pestle, and used to determine the preincubation TN and $\delta^{15}N$. These values were later used in the calculations as preincubation nitrogen content and isotopic composition.

Monitoring of physicochemical characteristics

In order to ensure that different trophic states were reached at both plots, fertilized and control, different environmental indicators related to eutrophication processes were monitored weekly during the experiment. The physicochemical characteristics of seawater were monitored just before the weekly refilling of the fertilizer pots. The salinity was measured in the water at seagrass canopy height using a WTW® Multi 3430 multiprobe.

Seawater for nutrient analysis was sampled during low tide at canopy height at 0, 10, 25, 50, and 100 cm distance from the terracotta pots. The water was immediately filtered through 0.45 μm pore size Minisart®-syringe filters into polyenthylene (PE) flasks, stored on ice, and frozen at −20°C until analysis at the Leibniz Centre for Tropical Marine Research (ZMT) in Germany. The nutrient concentrations in seawater were measured spectrophotometrically according to Grasshoff et al. (1999). The dry season sample measurements were made with a Skalar SAN++ System with detection limits of 0.03, 0.17, 0.22, 0.08, and 0.30 μM for nitrite (NO_2^-), NO_x ($NO_3^- + NO_2^-$), NH_4^+, (PO_4^{3-}), and silicate (Si $[OH]_4$), respectively. Wet season sample concentrations were measured with a Shimadzu UV-1700 UV/Vis spectrophotometer; the detection limits were 0.01, 0.08, 0.31, 0.06, and 0.11 μM for NO_2^-, NO_x ($NO_3^- + NO_2^-$), NH_4^+, PO_4^{3-}, and $Si(OH)_4$, respectively.

Chlorophyll *a* (Chl *a*) and total suspended matter (TSM) were analyzed from surface seawater above the experimental plots. Seawater samples were collected in PE containers of 10 liters volume, stored in the shade, and transported to the field laboratory. On the same day, the water samples were filtered through precombusted (5 h, 450°C) and preweighed Whatman® GF/F filters (≈ 0.7 μm pore size). The filters for the TSM analysis were subsequently dried at 40°C until constant weight and reweighed to estimate the TSM concentration (mg L^{-1}). The filters for Chl *a* analysis were stored frozen (−20°C) until analysis in the field laboratory. The Chl *a* was extracted from the filters in 5 mL 90% acetone for 24 h at 4°C in the dark and subsequently centrifuged for 3 min. The centrifugate was used to measure the Chl *a* concentration (μg L^{-1}) after Lorenzen (1967) using a HACH DR3900 spectrophotometer.

As an estimate of water clarity, we calculated the light attenuation coefficient. For that purpose, we measured

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photosynthetically active radiation (PAR) using a LI-COR LI-192 Underwater Quantum Sensor (LI-COR). The photosynthetic photon flux rates (μmol photons s^{-1} m^{-2}) were measured directly beneath the surface and at canopy height. The average of three different measurements of 15 s was used. The light attenuation coefficient K_d (m^{-1}) was calculated using the formula proposed by Kirk (1994):

$$K_d = \frac{(I_d/I_0)}{-d},$$

where I_0 and I_d are the measurements directly beneath the water surface and at seagrass canopy height, respectively, and d is the water depth (m) from the surface to the canopy height.

To estimate daily light availability (mol m^{-2} d^{-1}) and average temperature (°C) at both plots, in situ temperature and light availability were continuously logged every 10 min using Onset HOBO Pendant® Temperature/Light 64K data loggers. The loggers were installed at sediment surface level and were cleaned weekly. The light measurements were recorded in lux and converted to PAR as photon flux density (μmol photons s^{-1} m^{-2}) using the approximated conversion factor 51.2 (Valiela 1984, as cited in Carruthers et al. 2001) and multiplying the sum of all measurements of 1 d by 600 to account for the 10-min measuring intervals. Furthermore, we calculated the daylight hours from the light availability measurements.

Seagrass incubations

In both seasons, whole plants of all seagrass species present in the experimental (i.e., fertilized and control) plots were collected at the end of the fertilization experiment. All plants consisted of one shoot with leaves, a few roots, and a small piece of the rhizome. To create similar starting conditions, we placed the plants in a bucket in the dark with control seawater for 1 h before the incubation. We used 1.1-liter glass incubation chambers filled with seawater sampled close to the control site early in the morning. Aboveground and belowground tissues were incubated in the same chamber, as previous experiments with *Zostera noltei* showed no effect of rhizosphere oxygenation and inorganic nitrogen availability on leaf inorganic nitrogen uptake rates (Alexandre et al. 2010, 2011). In order to incubate similar amounts of seagrass biomass, 4 shoots of *T. hemprichii* and *C. serrulata*, 6 shoots of *C. rotundata*, and 8–10 shoots of *H. ovalis* and *H. uninervis* were used for the incubations. The different species were separately incubated in independent incubation chambers that acted as replicates ($n = 4$ per species).

Each incubation chamber was spiked with a preprepared solution of ^{15}N-labeled ammonium or nitrate. The solution was prepared with 98 atom% ^{15}N of $(^{15}NH_4)_2SO_4$ or $K^{15}NO_3$ (Sigma Aldrich). The final concentration was 2.5 μM of either $^{15}NH_4$ or $^{15}NO_3$, and nutrient concentrations present in the natural seawater. Incubations under light and dark conditions were performed simultaneously. Incubations of the light treatments were performed under ambient light conditions, while the incubation chambers of the dark conditions were wrapped in two layers of thick black plastic. This yielded a total of eight treatments (two trophic states, fertilized and control; two levels of light availability, light and dark; two N sources, NH_4^+ and NO_3^-) per season (dry and wet).

To avoid overheating, the chambers were placed in a large box filled with seawater, which acted as a water bath and was regularly monitored and exchanged with fresh seawater, so it did not exceed 30°C in the incubation chambers. During the experiment, the incubation chambers were shaken and rolled every 10 min to limit the build-up of a diffusion gradient. All incubations lasted 1 h to avoid bacterial remineralization (Hach et al. 2020). For logistic reasons, the incubations with plants from the different trophic states (i.e., fertilized and control) were made on 2 consecutive days, but always between 10:00 and 14:00.

Once the incubations had finished, the seagrass plants were removed from the chambers, rinsed with control seawater, and placed on ice to transport them. Once in the field laboratory, plants were processed (*see* "Experimental design and fertilization procedure" section). Water samples for nutrient concentration measurement were taken from the incubation chambers before and after incubation, the procedure was the same as for the other water nutrient samples (*see* "Monitoring of physicochemical characteristics" section).

Seagrass leaf biomass, leaf growth, and nitrogen demand

In order to estimate the seagrass leaf biomass, six 20 × 20 cm quadrats were installed in the fertilized plot and in the control plot, 3 weeks after the fertilization started in the dry season and 4 weeks after the fertilization started in the wet season. The six quadrats were placed approximately 50 cm from the center and 50 cm away from the edges of the experimental plot. Seagrass shoots inside the quadrats were marked at the basal part of the leaf near the sediment surface using the punch-hole method (Short and Duarte 2001). After 1 week, all seagrass shoots within the quadrats were collected and gently cleaned from epiphytes with seawater. All leaf biomass was dried at 40°C until constant weight, weighed, and subsequently homogenized with mortar and pestle for TN analysis (*see* "Nitrogen uptake calculation" section).

Relative daily growth rates (gDW^{-1} d^{-1}; DW = dry weight) were calculated for each species by dividing the newly grown biomass by the old leaf biomass and divided by the number of days between marking and harvesting (i.e., 7 d). The sum of new, old, and unmarked leaf biomass was converted to leaf biomass per square meter. The average seagrass leaf production per square meter was calculated from the sum of the

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relative daily growth rates normalized by the biomass per square meter.

The relative nitrogen demand (μM N gDW^{-1} d^{-1}) for the new leaf biomass was calculated by multiplying the relative growth rate with the TN of the new leaf biomass. The relative nitrogen demand multiplied by the leaf biomass per square meter gave the nitrogen demand per area (μM N m^{-2} d^{-1}).

Nitrogen uptake calculation

We calculated the species-specific leaf ^{15}N uptake (V_{15N}: expressed μM N g DW^{-1} h^{-1}) from the concentration of the ^{15}N in leaves by using $V15N = [FN \times (AFsample - AFbackground)]/MN \times t$, where F_N is the TN in the leaf sample (g N g^{-1} DW), AF_{sample} is the atom% ^{15}N in the seagrass leaf after the incubation, $AF_{background}$ is the atom% ^{15}N in the seagrass leaf prior to incubation, M_N is the molar mass of nitrogen (14×10^{-4} g μmol^{-1}), and t is the incubation time (in h). Plants sampled in the experimental plots (*see* "Experimental design and fertilization procedure" section) were used for background values in these calculations. The specific ^{15}N uptake rate was corrected for the inorganic nitrogen concentration in the natural seawater used for the incubations resulting in the N uptake rate. For simplicity, we assumed that the nutrient concentration remained constant during the incubations and the ^{15}N in NH_4^+ and NO_3^- of the natural seawater to be 0.366% like in air.

The nitrogen stable isotope composition of non-^{15}N-enriched seagrass leaf samples (i.e., background samples from "Experimental design and fertilization procedure" and "Seagrass leaf biomass, leaf growth, and nitrogen demand" sections) was analyzed in a Thermo Finnigan Delta Plus gas isotope ratio mass spectrometer coupled to a Flash 1112 EA elemental analyzer at the ZMT. ^{15}N-enriched samples were analyzed for their δ^{15}N and TN content at the Stable Isotope Facility at the University of California, Davis.

Statistical analyses

The impact of the fertilization treatment (control vs. fertilized) and season (dry vs. wet season) on the physicochemical parameters was tested. All environmental parameters measured in the wet season before the 6th of October, when it started raining, were excluded from the statistical analysis.

For the uptake rates, a full factorial design was not possible, as not all species were present in all seasons and treatments.

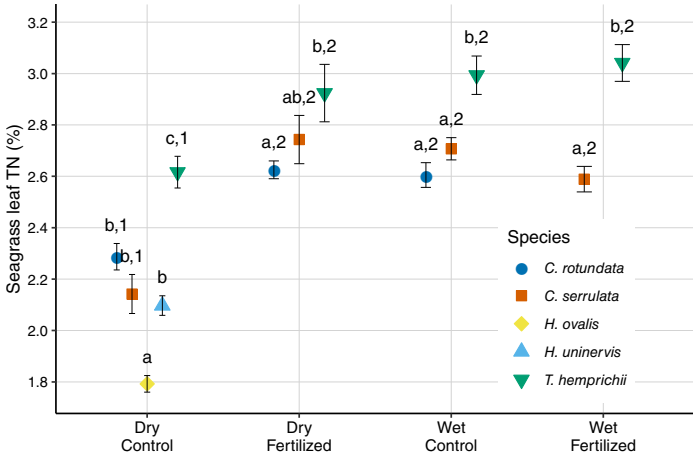


Fig. 2. Leaf TN (TN %, mean ± SE, $n = 3$) at the control and fertilized treatments in dry and wet season in 2017. Letters indicate significant differences among species in the same treatment and season, and numbers indicate intraspecific differences between treatments and season.

H. ovalis and *H. uninervis* were only present in the dry season in the control treatment. Therefore, for these species, differences in uptake rates between light (light vs. dark) and N source (NO_3^- and NH_4^+) were analyzed for the dry-control treatment only. For *C. rotundata*, not enough plants were found in the fertilized treatment in the wet season. Therefore, the effect of season and treatment were combined in the categories: dry-control, dry-enriched, and wet-control. Furthermore, differences in *C. rotundata* uptake rates between light (light vs. dark) and N source (NO_3^- and NH_4^+) were analyzed. Differences in NO_3^- and NH_4^+ uptake rates within the same season and treatment were compared as categorical variables between species (which ever present). Finally, we tested the differences in uptake rates of *C. rotundata*, *C. serrulata*, and *T. hemprichii* in a mixed-effect model with species as a categorical variable and N-type and season and treatment as a random factor.

Aboveground biomass and nitrogen demand per area were tested for differences between seasons as categorical variables only. Seagrass biomass can be heterogeneous across the meadow, and we did not quantify seagrass biomass before the fertilization. The initial differences in biomass could result in misleading conclusions. However, we tested for differences among species, seasons, and treatments as categorical variables in the relative nitrogen demand. Furthermore, we tested

Table 1. Seawater nutrient concentrations in the control and fertilized plots in the dry and wet seasons in 2017. Superscript letters identify differences between the means of the groups.

Season	Treatment	*n*	DIN (μM)	NH_4^+ (μM)	NO_x (μM)	NO_3 (μM)	PO_4 (μM)
Dry	Control	9	1.6 ± 0.3^{a}	1.2 ± 0.2^{ab}	0.4 ± 0.1^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}
	Fertilized	10	3.2 ± 0.8^{b}	2.1 ± 0.5^{c}	1.0 ± 0.3^{b}	0.9 ± 0.4^{b}	1.1 ± 0.8^{b}
Wet	Control	9	9.6 ± 0.7^{c}	1.0 ± 0.3^{a}	8.6 ± 0.8^{c}	8.2 ± 0.8^{c}	0.7 ± 0.1^{b}
	Fertilized	14	11.4 ± 0.8^{d}	2.2 ± 0.5^{bc}	9.1 ± 0.5^{d}	8.7 ± 0.5^{d}	0.8 ± 0.2^{b}

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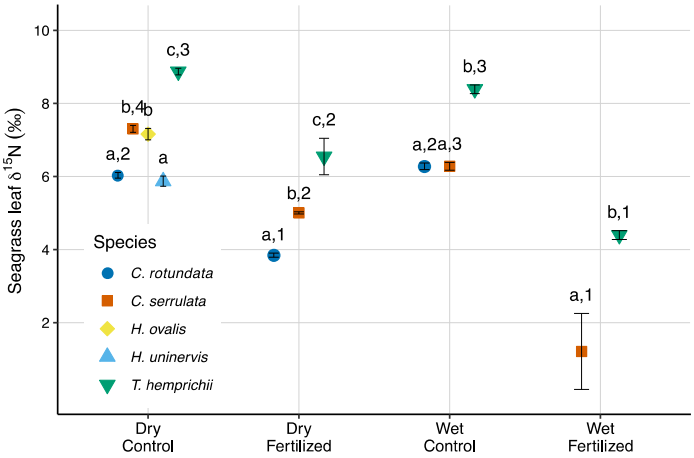


Fig. 3. Leaf nitrogen stable isotope composition (δ^{15}N, ‰, mean ± SE, $n = 3$) in dry and wet season at the control and fertilized treatment. Letters indicate significant differences among species in the same treatment and season, and the numbers indicate intraspecific differences between treatments and seasons.

differences in the nitrogen demand covered through inorganic nitrogen uptake by species, using the calculations for each season and treatment as replicates.

All comparisons were tested using generalized linear models (GLM). The models were fit using maximum likelihood and tested with Type II Wald χ^2 tests. Homoscedasticity of residuals of the linear models was confirmed by visual inspection of residual plots. Normality was tested using the Kolmogorov–Smirnov test. Afterwards, Tukey's post hoc tests were used to compare the means. As the residuals in the model for the PO_4 concentration were not normally distributed, a nonparametric Wilcoxon signed-rank test was used, followed by adjusting the p values using the Bonferroni method. All statistical analyses were carried out using R (R Core Team 2019). All values are given as mean ± SE. Letters identify significant differences (p-value < 0.05) between the means of the groups.

Results

Physicochemical characteristics during in situ nutrient enrichment

The conditions in the dry and wet seasons were markedly different. The light availability in the wet season was 80% lower than in the dry season (Supporting Information Table S1). Daylight hours were significantly higher in dry, 13.2 ± 0.1 h, than in wet season, 10.8 ± 0.3 h. The extinction coefficient of light (K_d) was significantly lower in the dry season (0.64 ± 0.05 m^{-1})

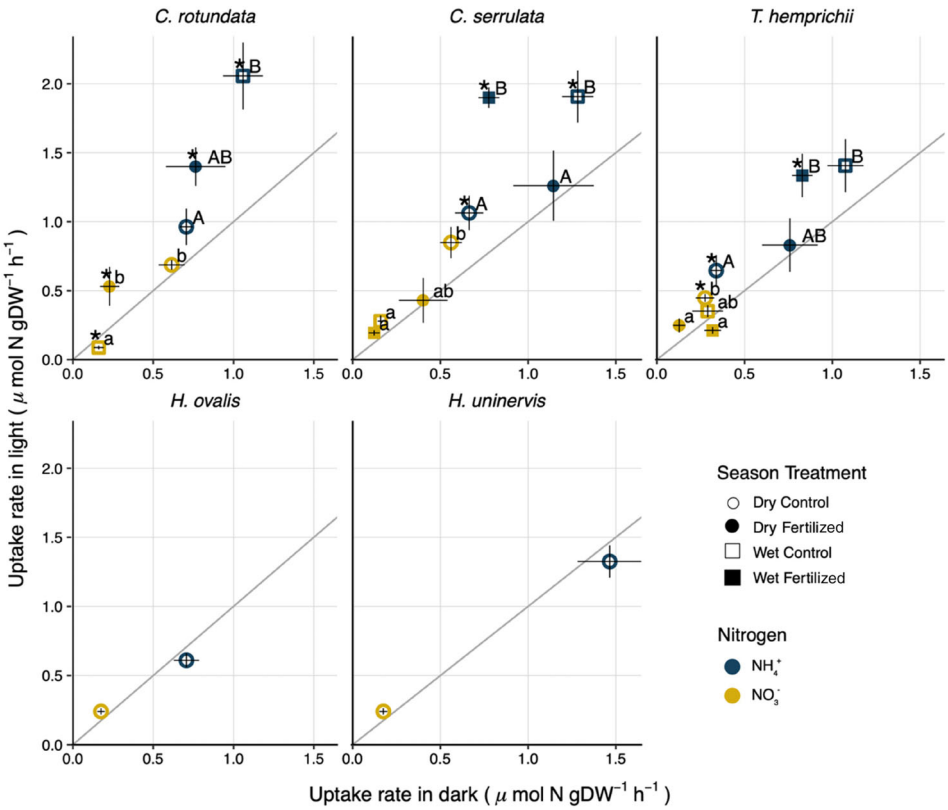


Fig. 4. Inorganic nitrogen uptake rates in light (μmol N gDW^{-1} h^{-1}, mean ± SE) plotted against the uptake rates in the dark in the five seagrass species within this study. Points located above the gray line indicate higher uptake rates in the light than in the dark. Asterisks indicate significant differences. Capital letters indicate significant differences in NH_4^+ uptake rates between seasons and treatments within the same species. Lower case letters indicate differences in NO_3^- uptake rates between seasons and treatments within the same species.

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than in the wet season ($2.22 \pm 0.90\ m^{-1}$), the same trend was observed for TSM (dry: $15.85 \pm 1.77\ mg\ L^{-1}$, wet: $39.11 \pm 2.97\ mg\ L^{-1}$) and Chl *a* concentration (dry: $0.58 \pm 0.13\ \mu g\ L^{-1}$; wet: $1.15 \pm 0.13\ \mu g\ L^{-1}$). Furthermore, temperature and salinity were significantly lower in the wet season than in the dry season (Supporting Information Tables S1, S2). Average temperatures were 29.3°C in the dry season and 28.5°C in the wet season with low daily variations. The dissolved oxygen concentration ($7.2 \pm 0.2\ mg\ L^{-1}$), pH (8.16 ± 0.0), and depth (0.88 ± 0.02 m) was not different between experimental plots.

Significant differences in nutrient concentrations were observed between control and fertilized plots (Table 1). In the case of nitrate, the concentration in control plots in the wet season was significantly higher (41-fold) than in the dry season. Although phosphate concentrations in the control plots were significantly higher (threefold) in the wet season than in the dry season, ammonium concentrations at the control and fertilized plots did not differ between seasons (Table 1; Supporting Information Table S2).

Seagrass leaf nitrogen content and stable isotope composition

The TN content in leaves of *C. rotundata*, *C. serrulata*, and *T. hemprichii* collected at the control plot in the dry season was ~20% significantly lower than in all other treatments regardless of the season (Fig. 2; Supporting Information Table S3). The TN content of *T. hemprichii* leaves was significantly higher than the other species in all seasons and treatments, except of *C. serrulata* leaves in the fertilized treatment in the dry season (Fig. 2). When present, *H. ovalis* leaves had the lowest TN concentration of all species (Fig. 2).

All species showed significantly lower leaf $\delta^{15}N$ values under the fertilized treatment compared to the control treatment of the same season (Fig. 3; Supporting Information Table S4). The fertilization in the dry season resulted in a 36% reduction of leaf $\delta^{15}N$ values in *C. rotundata*, a 31% reduction in *C. serrulata*, and a 26% reduction in *T. hemprichii*. In the wet season, the differences between treatments were even higher than in the dry season, the average leaf $\delta^{15}N$ values in the fertilized treatment were 81% and 48% lower than in the control treatment in *C. serrulata* and *T. hemprichii*, respectively (Fig. 3). As for TN leaf content, $\delta^{15}N$ values in *T. hemprichii* leaves were higher than for the other species in the same treatment. *C. serrulata* had higher $\delta^{15}N$ values in leaves than *C. rotundata* in both treatments in the dry season (Fig. 3; Supporting Information Table S4).

Inorganic nitrogen leaf uptake rates

The concentration of the ^{15}N-labeled nitrogen (either NH_4^+ or NO_3^-) added at the start of all incubations was always 2.5 μM. However, the natural seawater used for the incubations also contained inorganic nitrogen; since the incubations were done on consecutive days, the concentrations varied (*see* Supporting Information Table S5). In the dry season, the background nitrogen concentrations were $0.7 \pm 0.35\ \mu M\ NH_4^+$ and $0.36 \pm 0.21\ \mu M\ NO_3^-$ before the addition of the tracer for the incubations with plants from the control treatment, and $2.04 \pm 1.19\ \mu M\ NH_4^+$ and $0.74 \pm 0.37\ \mu M\ NO_3^-$ for the plants from the fertilized treatment. In the wet season, the initial nitrogen concentrations before the addition of the tracer were $1.23 \pm 0.71\ \mu M\ NH_4^+$ and $7.17 \pm 1.37\ \mu M\ NO_3^-$ for the control treatment, and $1.5 \pm 0.87\ \mu M\ NH_4^+$ and $7.41 \pm 0.39\ \mu M\ NO_3^-$ for the fertilized treatment.

All species showed significantly higher NH_4^+ uptake rates in the wet season than in the dry season (Fig. 4). *C. rotundata* and *C. serrulata* in the control treatment showed seven and three times higher NO_3^- uptake rates in dry than in wet season (Fig. 4). The trophic state had no significant effect on uptake rates regardless of the season and nitrogen source. Only *C. serrulata* and *T. hemprichii* grown at the fertilized plot in the dry season showed NO_3^- uptake rates significantly lower than at the control site (Fig. 4; Supporting Information Table S6). All seagrass species could take up both nitrogen species

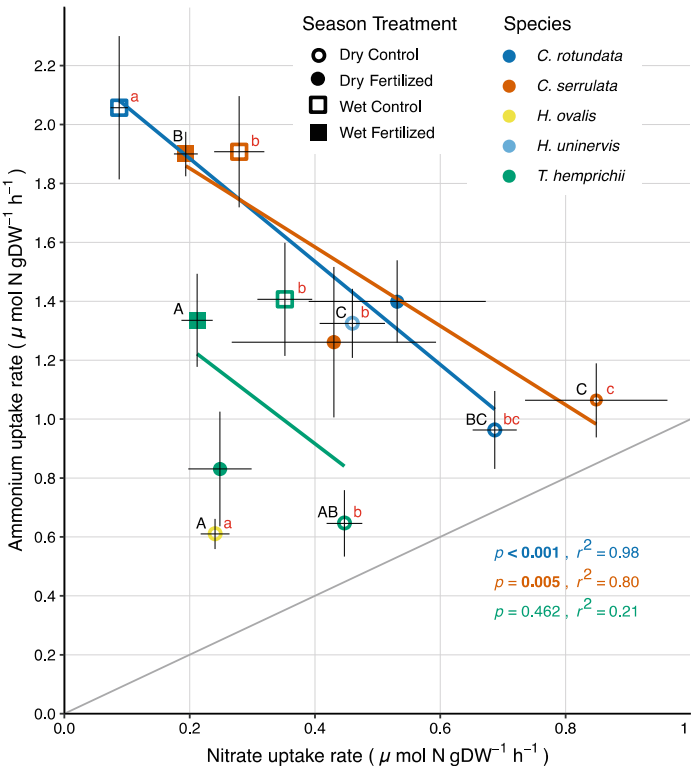


Fig. 5. Seagrass leaf ammonium vs. nitrate uptake rates in the light (μmol N gDW^{-1} h^{-1}). Capital black letters indicate significant differences in NH_4^+ uptake rates among species within the same season and treatment. Lower case red letters indicate differences in NO_3^- uptake rates among species in the same season and treatment. No letters indicate no differences in inorganic nitrogen uptake rates among species' uptake rates within the same nitrogen source. The regression lines indicate the correlation of NH_4^+ uptake and NO_3^- uptake within seagrass species. Gray line represents line 1 : 1.

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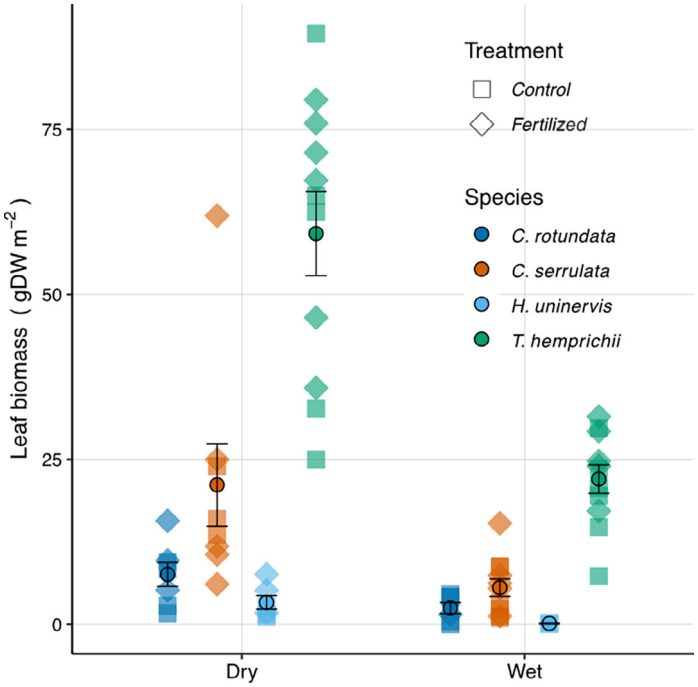


Fig. 6. Leaf biomass measurements of the different seagrass species (different colors) grown under fertilized and control treatments (different symbols) in the dry and wet season. The average (± SE) is shown in the framed round symbols.

through leaves under light and darkness conditions (Fig. 4). Uptake rates tended to be higher in the light; however, often, this was not significant, and there were no systematic differences between uptake rates in light and dark incubations (Fig. 4). NO_3^- uptake rates in light were significantly higher than in the dark for *C. rotundata* grown in the fertilized treatment in the dry season and significantly lower in the control treatment in the wet season. *T. hemprichii* NO_3^- uptake rates were significantly higher in light than in the dark in plants grown at the control site in the dry season. In the wet season, NH_4^+ uptake rates were significantly higher in light than in darkness, except for *T. hemprichii* in the control treatment. In the dry season, NH_4^+ uptake rates were significantly higher in light than in the dark for *C. rotundata* in the fertilized treatment and *T. hemprichii* in the control treatment. Overall, the NH_4^+ uptake rates in the dark were 20% lower in the dry season and 40% lower in the wet season than in light, while differences in NO_3^- uptake rates between light and dark were smaller.

Under light conditions, NH_4^+ uptake rates were consistently higher than NO_3^- uptake rates (Fig. 5). However, these differences were not significant for *C. rotundata*, *C. serrulata*, and *T. hemprichii* grown in the control treatment in the dry season. The lowest NO_3^- uptake rates were observed in *C. rotundata* grown at the control site during the wet season, followed by *T. hemprichii* and *C. serrulata* grown in the same conditions (Fig. 5). The highest NO_3^- uptake rates were measured for *C. serrulata* grown in the control treatment during the dry season. Contrary to NO_3^-, the lowest uptake rates of NH_4^+ were observed in *H. ovalis* and *T. hemprichii* at the control treatment in the dry season, followed by *C. rotundata*, *C. serrulata*, and *H. uninervis*, which had significantly higher uptake rates.

No differences among uptake rates of *C. rotundata*, *C. serrulata*, and *T. hemprichii* were detected at the fertilized treatment grown in the dry season (Fig. 5; Supporting Information Table S7). In the wet season, there was an interaction between seagrass species and nitrogen source in plants grown in the control treatment, showing similar NH_4^+ uptake rates of *C. rotundata*, *C. serrulata*, and *T. hemprichii*, but significantly lower NO_3^- uptake rates in

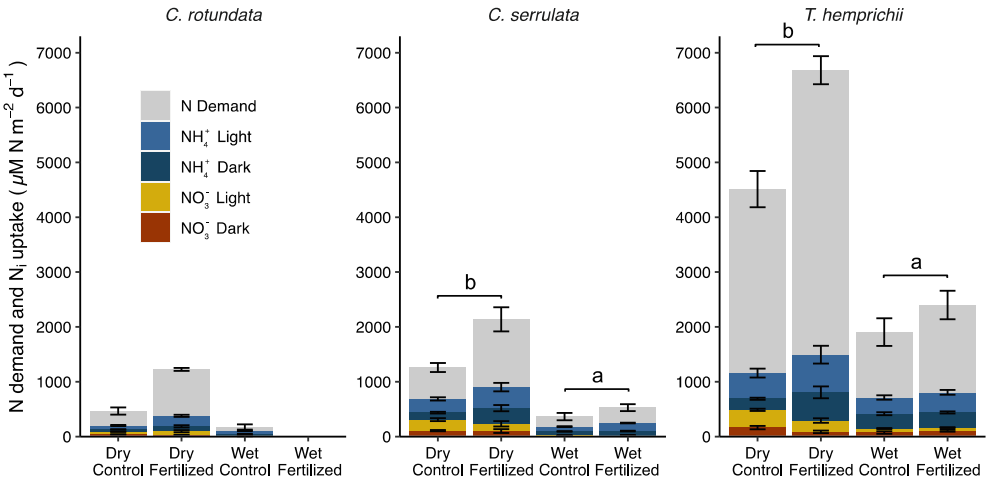


Fig. 7. Daily nitrogen demand for leaf growth and N_i leaf uptake rates of the different inorganic sources, normalized to the area. The gray bars indicate the daily nitrogen demand (μM N m^{-2} d^{-1}) calculated from normalized leaf growth and the nitrogen content of the new leaf material. The stacked bars are the daily inorganic nitrogen uptake rates through seagrass leaves calculated from the seagrass incubations. Letters indicate significant differences in nitrogen demand between the different treatments within the same species.

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C. rotundata than in *C. serrulata* and *T. hemprichii*. On the contrary, in the fertilized treatment, NO_3^- uptake rates of *C. serrulata* and *T. hemprichii* were similarly low, while *C. serrulata* had higher NH_4^+ uptake rates than *T. hemprichii* (Fig. 5; Supporting Information Table S7). *C. rotundata* and *C. serrulata* NO_3^- uptake rates were significantly and negatively correlated to NH_4^+ uptake rates, while this correlation was not significant for *T. hemprichii* (Fig. 5).

Seagrass leaf biomass, leaf growth, and nitrogen required for growth

The following results are limited to measurements for *C. rotundata*, *C. serrulata*, and *T. hemprichii* since *H. ovalis* and *H. uninervis* were not sufficiently abundant to reliably measure leaf growth (i.e., they were present in less than three quadrats used to quantify leaf growth); the same happened with *C. rotundata* in the fertilized treatment in the wet season. The average leaf biomass, as the sum of *C. rotundata*, *C. serrulata*, and *T. hemprichii* regardless of the fertilization treatment, was 54.2 gDW m^{-2} with seasonal variations. In the dry season, the average leaf biomass as the sum of all species was ~ 3 times significantly higher compared to the wet season (Supporting Information Table S8). This was significant for all species found in both seasons regardless of the treatment (Supporting Information Table S8; Fig. 6). In both seasons, *T. hemprichii* showed the highest leaf biomass (Fig. 6).

Average seagrass leaf production as the sum of all species per area was also significantly higher in dry (1.5 ± 0.3 gDW m^{-2} d^{-1}) than in wet season (0.8 ± 0.2 gDW m^{-2} d^{-1}) ($p = 0.0024$). Leaf production was significantly correlated to standing leaf biomass across treatments and seasons (Supporting Information Fig. S1). However, if leaf production and nitrogen demand for new leaf biomass are normalized by the standing seagrass leaf biomass, no differences in nitrogen demand (Supporting Information Fig. S2) and leaf production (Supporting Information Table S9) among seasons, treatments, and species were detected. The nitrogen demand covered by NO_3^- uptake tended to be the highest in all species grown in the dry season in the control treatment (Fig. 5; Supporting Information Fig. S2). On average, *T. hemprichii* covered 30% of its nitrogen demand for leaf growth through inorganic nitrogen uptake by leaves; this is significantly less than the 45% ($p = 0.043$) covered by *C. rotundata* and 48% ($p = 0.010$) covered by *C. serrulata* (Supporting Information Table S10; Fig. S2). The nitrogen demand per area was higher in the dry than in the wet season for *C. serrulata* ($p = 0.0094$) and *T. hemprichii* ($p = 0.0086$; Fig. 7).

Discussion

Our study showed that both inorganic nitrogen species are taken up through leaves by all seagrass species in the light and darkness. Uptake of NH_4^+ and NO_3^- was negatively correlated with a strong preference for NH_4^+ at higher nitrogen availability. The nutrient enrichment had a significant positive effect on the nutrient concentration in the water in both seasons and on the leaf nitrogen concentrations in dry season. However, eutrophication in the wet season had a stronger effect on inorganic nitrogen uptake rates than the fertilization alone. Leaf production did not differ among species, seasons, or treatments, if normalized by standing leaf biomass. Overall, *T. hemprichii* leaf uptake rates were lower than those of *C. rotundata* and *C. serrulata*. Consequently, these latter species covered up to 50% of their daily nitrogen demand for leaf growth while, *T. hemprichii* only covered up to 30%.

Meadow trophic status affects nitrogen content and isotopic composition in seagrass leaves

The low environmental nutrient availability in the control treatment in the dry season was reflected in all species' leaf nitrogen content. The observed dissolved inorganic nitrogen values close to 0 suggest a potential nitrogen limitation of plants within this treatment in this season. Under these conditions, fertilized seagrass often show higher leaf nitrogen content than in the control treatment, as also observed in other studies with tropical climax species (Agawin et al. 1996; Terrados et al. 1999). This was not the case for the rest of the treatments in our study, probably because ambient seawater nitrogen availability was high during the wet season, suggesting that nitrogen was not a limiting factor during this season.

Similar leaf nitrogen concentrations in *C. rotundata* and *T. hemprichii* were reported in a fertilization experiment in the Philippines (Agawin et al. 1996). Higher leaf nitrogen concentrations in *T. hemprichii* than in the other species were also reported from the Philippines and the Red Sea (Agawin et al. 1996; Duarte et al. 2018). This has also been observed in belowground tissues in which *T. hemprichii* had a higher N content than *C. serrulata* and *Halophila stipulacea* (Viana et al. 2020). This is consistent with the persistent life history strategy of *T. hemprichii*, which includes the storage of nitrogen, rather than immediately investing the nitrogen for growth, typical for small and fast-growing species like *H. ovalis*.

Lower leaf $\delta^{15}N$ values in the fertilized treatment than in the control treatment within the same season indicate that the plants readily took up the nitrogen from the fertilizer. Surprisingly, in the wet season when ambient nitrate concentrations were high, the preference for nitrogen from the artificial fertilizer was even higher, as seen in the lower $\delta^{15}N$ values in *T. hemprichii* and *C. serrulata* leaves when comparing the fertilized treatments between the seasons (Fig. 3). In both seasons, NH_4^+ from the fertilizer amounted to $\sim 50\%$ of the NH_4^+ available (Table 1). The higher preference could indicate a substrate feedback mechanism as shown in *Z. noltei*, where the presence of NO_3^- increases NH_4^+ uptake and inhibits NO_3^- uptake (Alexandre et al. 2010).

The meadow trophic status and isotopic signature of the available nitrogen were apparent in the nitrogen content and

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$\delta^{15}N$ in seagrass leaves of all species, especially in *T. hemprichii*. This implies that relative to its nitrogen content, *T. hemprichii* took up less of the artificial fertilizer and therefore has a lower nitrogen filter function than both *Cymodocea* species.

Influence of trophic state on leaf N_i uptake rates

Our first hypothesis was verified; C. *rotundata* and *C. serrulata* acquired less NO_3^- in under high nitrogen concentrations, that is, in both treatments in the wet season compared to the control treatment in the dry season. Simultaneously both species acquired more NH_4^+ through leaf uptake in the wet season than in the dry season. NH_4^+ and NO_3^- uptake rates were negatively correlated. In previous experiments investigating NH_4^+ and NO_3^- uptake kinetics, often the inorganic nitrogen source is supplied alone; in that case, the uptake usually follows the Michaelis–Menten kinetics. However, if both nitrogen species are abundant, a preference for NH_4^+ over other nitrogen forms has been reported for various seagrass species (Alexandre et al. 2010, 2011, 2015; Viana et al. 2019).

NH_4^+ uptake is energetically more efficient than NO_3^- uptake (Touchette and Burkholder 2000). A proposed downregulation mechanism of NO_3^- uptake is that the presence of NH_4^+ or one of its products from assimilation suppresses the nitrate reductase activity (Alexandre et al. 2010) or the active membrane transport of NO_3^- (Iizumi and Hattori 1982). Furthermore, nitrate assimilation requires an active transport system with specific transport proteins. In our case, the fertilized plants, accustomed to constantly high NH_4^+ concentrations, could have fewer active NO_3^- transporters (Touchette and Burkholder 2000).

The reduction of NO_3^- uptake and the increase in NH_4^+ uptake seem to be concentration dependent. Higher NH_4^+ and lower NO_3^- in this study were only significant when comparing uptake rates in dry season to wet season with typhoon-induced eutrophication. For *Z. noltei*, similar observations were made, no uptake inhibition was observed at substrate concentrations $< 5\,\mu M$ (La Nafie et al. 2014), but substrate concentrations of $5\,\mu M$ NO_3^- and NH_4^+ each had a negative effect on NO_3^- uptake rates (Alexandre et al. 2010).

Furthermore, high NO_3^- concentrations may downregulate NO_3^- uptake rates (Sandoval-Gil et al. 2015, 2016). In our study, the NO_3^- uptake rates are suppressed at ambient NO_3^- concentrations of $\sim 9\,\mu M$ for *C. rotundata* and *C. serrulata* in the wet season. In *Z. marina* in Mexico, NH_4^+ concentrations $> 5\,\mu M$ resulting from oyster farm effluents led to a locally higher leaf NH_4^+ uptake affinity for NH_4^+ and primary production in *Z. marina* (Sandoval-Gil et al. 2015, 2016).

Influence of light on N_i uptake

Since inorganic nitrogen uptake and assimilation requires energy and carbon skeletons, these processes are dependent on photosynthesis or energy and carbon reserves (Touchette and Burkholder 2000, 2007; Alexandre et al. 2015). Different seagrass species can sustain NH_4^+ and NO_3^- uptake through leaves and NH_4^+ uptake through roots in the darkness (Iizumi and Hattori 1982; Lee and Dunton 1999; Alexandre et al. 2015).

In this study, NH_4^+ uptake rates were consistently lower in the dark than in the light; this trend was stronger and significant during eutrophication in the wet season. After 3 weeks of light limitation, the carbon reserves in the leaves could have been exhausted, and therefore NH_4^+ assimilation was reduced in darkness, while during active photosynthesis, the seagrasses maintained NH_4^+ assimilation. In the wet season, when NO_3^- uptake rates were already low no further reduction was detected. All species acquired NO_3^- and NH_4^+ after 2 h of darkness. Longer preincubations in darkness and indicators of tissue carbon metabolism and enzymatic activity could elucidate the underlying processes and differences between species.

Standing leaf biomass controls leaf production and nitrogen demand

Threefold higher standing leaf biomass supported a higher leaf production per area in the dry season. However, if the available photosynthetic active tissue is considered, all seagrass species produced a similar amount of new leaf biomass in all treatments. Relative daily growth rates of *C. rotundata*, *C. serrulata*, and *T. hemprichii* were in the same range or higher than in other studies (Heijs 1985; Erftemeijer and Herman 1994; Vermaat et al. 1995; Udy and Dennison 1997; Uku and Björk 2005) indicating nonlimiting nutrient conditions in this study.

Our third hypothesis was not confirmed; even though *T. hemprichii* had higher TN values in the leaves, it had neither a higher relative nitrogen demand nor higher nitrogen uptake rates than the other species. However, as *T. hemprichii* had the highest biomass of all species in this multispecies meadow (Thomsen et al. 2020), it had the highest nitrogen demand per area. Seasonal and annual fluctuations in seagrass leaf biomass are common, and leaf biomass in this study falls in the same range as in meadows of the same species in Indonesia and Kenya (Erftemeijer and Herman 1994; Uku and Björk 2005). However, we found that seagrass biomass drives the nitrogen filtering capacity, which therefore also underlies these variations. The reduction of seagrass leaf biomass at the study site by 75% between 2009 and 2017 (Thomsen et al. 2020) implies a similar reduction in nitrogen filtering capacity.

The nitrogen filter function is species specific

Internal recycling and uptake from belowground reduce the nitrogen filter function of seagrasses. In our study, *T. hemprichii* covers $\sim 15\%$ less of its nitrogen requirements through leaves than *C. rotundata* and *C. serrulata*. In addition, it had a higher TN content and a higher $\delta^{15}N$ in the leaves than the *Cymodocea* species. Possibly, *T. hemprichii* relies on additional nitrogen sources or higher internal recycling, it therefore has a lower filter function. A monospecific

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T. hemprichii meadow with the same seagrass biomass would therefore have a lower filter function than a *C. rotundata* and/or *C. serrulata* meadow.

We underestimate the nitrogen demand of all species, as whole plant production may be up to an order of magnitude higher than aboveground production only (Tomasko and Dunton 1995). Furthermore, with only considering leaf nitrogen demand, we underestimate interspecific differences in the inorganic nitrogen filter function, because we do not account for different growth strategies. Although *E. acoroides* and *T. hemprichii* allocate 90–95% of their nitrogen demand for leaf growth, *C. rotundata* and *H. uninervis* have shown higher horizontal apical growth (Erftemeijer et al. 1993; Marbà et al. 2002). Therefore, this method underestimates the nitrogen demand of *C. rotundata* more than of *T. hemprichii*.

Nutrient resorption from senescent leaves can contribute ~ 10% of the plants' nitrogen demand. The resorption of *T. hemprichii* and *E. acoroides* can be higher than in other species because translocation within one ramet is more likely than the translocation between ramets (Stapel and Hemminga 1997). *T. hemprichii* can cover up to 28% of its nitrogen demand for leaf growth through internal reuse (Stapel et al. 2001). Overall, the nitrogen filter function is species specific, with *T. hemprichii* having a lower nitrogen filter function than the other species investigated in this experiment.

Eutrophication in a seagrass meadow: A self-reinforcing process

Seagrasses acclimate physiologically and morphologically to lower their energy and carbon demand as a reaction to light limitation and eutrophication. Lower growth, narrower and shorter leaves, and fewer leaves per shoot are the result (Lee and Dunton 1997; Ruiz and Romero 2001, this study). The eutrophication reduced the seagrass leaf growth and leaf biomass by area after only 4 weeks in the wet season, therefore reducing their nitrogen filter function (Fig. 7). This implies that eutrophication becomes a self-reinforcing process in the short-term, once above a certain threshold.

In the long-term, the eutrophication from aquaculture effluents resulted in large-scale seagrass declines in Hainan (Thomsen et al. 2020). Interestingly, *T. hemprichii* is the species that remained as one of the last species after decades of eutrophication from aquaculture effluents (Herbeck et al. 2014; Thomsen et al. 2020). By inducing seagrass declines and shifting the species composition toward *T. hemprichii* monospecific meadows, eutrophication becomes a self-reinforcing process also in the long term.

Potential inorganic nitrogen filter function in Hainan

Overall, the nitrogen filter function, which mitigates eutrophication, was driven by seagrass biomass. However, seagrass leaf biomass at our study site was low compared to earlier measurements in the same area (Herbeck et al. 2014; Thomsen et al. 2020). Furthermore, the aboveground seagrass biomass underlies strong seasonal variations (Herbeck et al. 2014; Thomsen et al. 2020), implying seasonal variation in the nitrogen filter function.

Apart from the strong seasonality, the nitrogen discharges exceed the seagrass nitrogen filter function in NE Hainan. Annually, 103.6 tons of DIN are discharged from aquaculture ponds into the coastal waters (Herbeck and Unger 2013). With an average uptake rate of 34.6 μM N gDW^{-1} d^{-1} across the three species and a moderate seagrass leaf biomass of 54.2 g DW m^{-2} as recorded in this study, the meadow would need to be 10.2 km^2 large to remove the inorganic nitrogen discharges from the aquaculture ponds. With 33.1 km^2 backreef area in NE Hainan (Herbeck and Unger 2013), shallow enough for potential seagrass growth, the inorganic nitrogen from aquaculture effluents could potentially be removed. However, considering strong, eutrophication induced, seagrass biomass loss of 87% between 2008 and 2017 (Thomsen et al. 2020) the eutrophication-mitigation effect of the damaged meadows is likely to be minimal. With the loss of the seagrass and its nitrogen filter function, further eutrophication above the threshold of 8 μM DIN calculated for the region (Thomsen et al. 2020) is likely to cause further seagrass losses. Furthermore, only 21% of the nitrogen discharged from the aquaculture ponds is dissolved inorganic nitrogen. In addition, 77 tons of dissolved organic nitrogen and 320 tons of particulate nitrogen are discharged, annually (Herbeck and Unger 2013). Further studies on organic nitrogen uptake and the nitrogen filter function on the ecosystem level are needed to fully quantify the nitrogen filter function.

Overall, our findings suggest that once eutrophication surpasses the nitrogen filter function of seagrasses and negatively impacts seagrass growth, their eutrophication mitigation potential is diminished, which further amplifies eutrophication. This implication in general helps to understand why eutrophication is one of the major threats to seagrasses, globally.

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