N$_2$ fixation and primary productivity in a red sea *Halophila stipulacea* meadow exposed to seasonality

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Abstract

The tropical seagrass *Halophila stipulacea* exhibits a limited capacity to use nitrate, but has a high uptake capacity and efficiency for ammonium. Consequently, N$_2$ fixation by associated diazotrophic epiphytes may be important in providing ammonium needed for seagrass photosynthesis and growth. When exposed to seasonality, this association could provide a competitive advantage for *H. stipulacea* in relation to other seagrass species, but related knowledge is scarce. Here, we thus report measurements of net photosynthesis (O$_2$ fluxes) and N$_2$ fixation (acetylene reduction) associated with *H. stipulacea* from the Northern Red Sea over all four seasons of 2013. In parallel, we characterized the seagrass meadow areal extent, shoot and leaves density, and the leaf area index. Using this data, we quantified meadow net primary production and N$_2$ fixation and estimated the photo-metabolic N demand met by N$_2$ fixation at the community level. Results revealed a marked seasonality of meadow N$_2$ fixation, with rates ranging from 0.06 in winter to 4.60 mmol N m^{-2} d^{-1} in summer, i.e., an increase by two orders of magnitude. In summer, when nutrient concentrations in the water column were lowest and light intensity and temperature highest, there was a significant positive linear relationship between meadow net primary production and N$_2$ fixation, with the latter accounting for ca. 20% of the photo-metabolic N demand. These findings suggest that N$_2$ fixation by associated diazotrophic epiphytes can mitigate N limitation of *H. stipulacea* meadows under N scarcity. This trait may also favor the ongoing geographical expansion of *H. stipulacea*.

High photosynthetic benthic primary production represents a key ecosystem service provided by seagrass systems worldwide, as it underpins their capacity to store large amounts of carbon (Duarte et al. 2005; Fourqurean et al. 2012). However, tropical seagrass meadows often grow in highly oligotrophic environments, where primary productivity is usually nutrient-limited (Duarte 1995). Seagrass productivity and growth in tropical environments is mainly limited by nitrogen (N), phosphorus (P), or both (Armitage et al. 2005; den Haan et al. 2013; Ferdie and Fourqurean 2004; Johnson et al. 2006; Lee and Dunton 2000; Powell et al. 1989; Short et al. 1990; Udy and Dennison 1997; Udy

et al. 1999). Given the limited availability of nutrients, seagrasses run a tight internal nutrient economy (Hemminga et al. 1999), with nutrients conserved by recycling and transfer from old to young growing leaves (e.g., Pedersen et al. 1997) as well as between ramets, i.e., the seagrass modular units (e.g., Marbà et al. 2002). In this context, any input of new N helps to mitigate nutrient limitation and favors the productivity of these ecosystems and their ability to act as a carbon (C) sink. One important source of N comes from dinitrogen (N$_2$)-fixing prokaryotes (diazotrophs) associated with the plant. As such, N$_2$ fixation has been extensively studied in seagrass habitats (Blackburn et al. 1994; Capone et al. 1979; Capone and Taylor 1980; Hamisi et al. 2009; McRoy et al. 1973; Moriarty and O'Donohue 1993; O'Donohue et al. 1991; Patriquin and Knowles 1972; Pereg-Gerk et al. 2002; Welsh et al. 1996a,b). N$_2$ fixation rates are generally higher in tropical seagrasses and comparable to those observed in cyanobacterial mats (*see* Cardini et al. 2014 and Welsh 2000 for reviews). This indicates that N$_2$ fixation is an ecologically relevant and quantitatively significant process

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associated with tropical seagrass meadows. In rhizosphere sediments, N_2 fixation rates correlate well with sulphate reduction (Capone 1982; Welsh et al. 1996c). In fact, sulphate reducing bacteria are closely associated with seagrass roots (Nielsen et al. 2001), and these bacteria can be responsible for the bulk of nitrogenase activity in seagrass sediments (Welsh et al. 1996a; Sun et al. 2015). However, epiphytic N_2 fixation on seagrass leaves often occurs at high rates and can also account for an important portion of total N_2 fixation in these ecosystems (Goering and Parker 1972; Capone and Taylor 1977; Capone et al. 1979; Hamisi et al. 2009). In many seagrass species, uptake affinities for ammonium are higher in leaves than in roots (Touchette and Burkholder 2000). The importance of leaf N uptake varies with circumstances, but can represent a major share of N uptake by seagrasses (Stapel et al. 1996; Pedersen et al. 1997; Terrados and Williams 1997). Leaf N uptake by adsorption and absorption is rapid and concentration-dependent (Short and McRoy 1984; Pedersen et al. 1997) and can thus be very important in oligotrophic environments for seagrasses to be able to quickly benefit from short-lived nutrient pulses. However, research efforts on phyllosphere N_2 fixation in tropical seagrass meadows have recently been minimal. Moreover, there is little understanding as to what extent this process can facilitate the productivity of seagrass meadows exposed to changing environmental conditions. The present study aims to contribute in this context.

Halophila stipulacea is a tropical and sub-tropical seagrass species widely distributed in the Indian Ocean and the Red Sea (Larkum and den Hartog 1989). However, it colonized the Eastern Mediterranean basin following the opening of the Suez Canal, and from there it spread into the Southern and Western Mediterranean (van der Velde and den Hartog 1992; Boudouresque and Verlaque 2002; Gambi et al. 2009; Sghaier et al. 2011). Now, there are several reports that indicate its geographical expansion into the Eastern Caribbean (Ruiz and Ballantine 2004; Willette and Ambrose 2009, 2012). In these areas, *H. stipulacea* acts as an invasive species by outcompeting other native seagrasses such as *Syringodium filiforme* (Willette and Ambrose 2012) and endemic species such as *Posidonia oceanica* (Gambi et al. 2009). The reasons for the success of *H. stipulacea* are not clear; however, the N acquisition strategy of this species, characterized by high capacity and efficiency for ammonium uptake, may favor the geographical expansion and potential invasiveness of *H. stipulacea* into areas with variable N concentrations (Alexandre et al. 2014; van Tussenbroek et al. 2016). In this context, N_2 fixation and ammonium production by associated diazotrophs could play an important role.

Early work by Wahbeh and colleagues focused on *H. stipulacea* in Aqaba, Jordan (Wahbeh 1983, 1984a,b, 1988; Wahbeh and Mahasneh 1984, 1985). Pereg and colleagues (Pereg et al. 1994; Pereg-Gerk et al. 2002) investigated N_2 fixation activity associated with *H. stipulacea* in Eilat, Israel. The present study aims to understand further the role of N_2 fixation in *H. stipulacea* from the Gulf of Aqaba building on this body of existing knowledge. In particular, we made use of the high seasonality that characterizes the Gulf of Aqaba to fill the gaps in knowledge regarding the seasonality of N_2 fixation and primary productivity in *H. stipulacea* and the link between these two processes in relation to changes in external environmental conditions. This information may help to explain the ability of *H. stipulacea* to adapt to pronounced variations in environmental N availability and to colonize and invade new (temperate) habitats.

Materials and methods

Site description and seagrass characterization

This study was conducted at the Marine Science Station (MSS) of the University of Jordan, in the Northern Gulf of Aqaba (29° 27′ N, 34° 58′ E) (Fig. 1). Here, *H. stipulacea* forms a monospecific seagrass meadow in front of the pier of the Marine Science Station along a gentle water depth gradient on a substrate characterized by silicate sediment of terrigeneous influence and composed primarily of quartz sand (Rasheed et al. 2003). The seagrass meadow is framed northward by small reef carbonate structures in the vicinity of the container port of the city of Aqaba, and southward by a fringing coral reef protected by a marine reserve. Meadow and plant features of the *H. stipulacea* meadow were assessed using SCUBA in November 2013. The meadow surface area was estimated based on the interpretation of high-resolution satellite images (© Digital Globe) with ground-truthing conducted using measuring tapes laid along the borders of the meadow. To estimate shoot density, the number of shoots was counted in random quadrats (10×10 cm, $n = 60$) along the entire water depth gradient from ~ 5 m to ~ 30 m, and the resulting mean number of shoots was normalized to m^{-2} benthic area. To estimate the number of leaves m^{-2}, shoot density was multiplied by the average number of 8 leaves per shoot (Wahbeh 1984a). Shoots were also collected haphazardly during all seasons of 2013 ($n = 8$ per season) and taken directly to the laboratory for analysis. For each shoot, the length and width of each leaf was recorded in ImageJ (Schneider et al. 2012) using planar photographs, and the leaf area index (LAI) was calculated using the one-sided leaf area (approximated to a rectangle).

The study site experiences strong seasonality reflected by substantial variability of key environmental parameters throughout the year, caused by the annual water column thermal stratification cycle that characterizes the Gulf of Aqaba (Carlson et al. 2014). Thermal stratification in the Gulf of Aqaba develops from May to November with a maximum during August/September, while deep-water mixing occurs from January to April reaching the maximum mixing depth in March/April (Carlson et al. 2014). Thus, in order to study the effect of seasonality on N_2 fixation and primary productivity by the seagrass and its epiphytic microbial
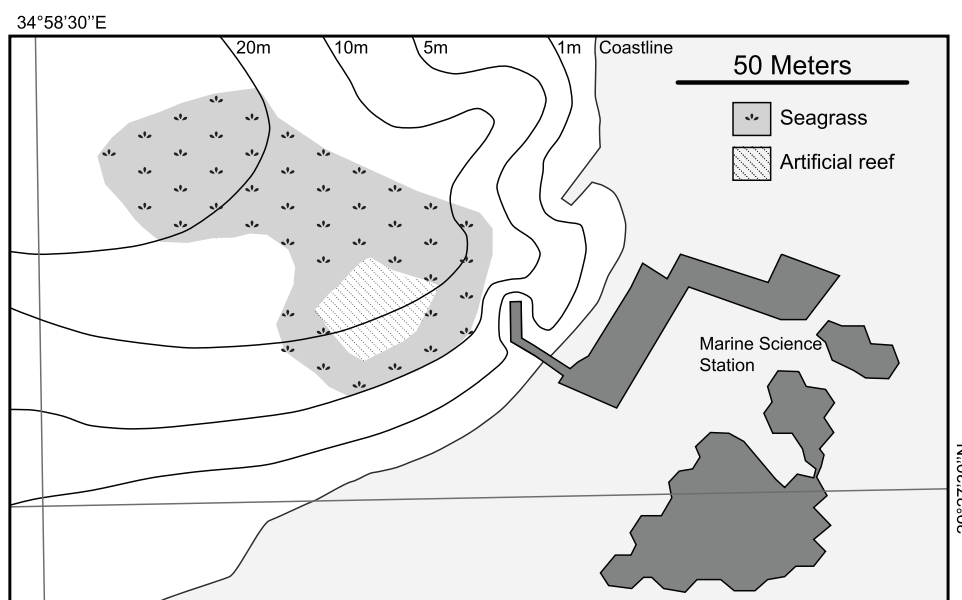
Fig. 1. *H. stipulacea* meadow in front of the Marine Science Station (Aqaba, Jordan).

communities, experiments described below were conducted once in each of the following months representing a respective season: February (winter), April (spring), September (summer), and November (fall) during the year 2013.

Environmental monitoring

Key environmental parameters were monitored during all seasons at the study site at 10 m water depth. In situ light intensity (lux) and temperature (°C) were continuously recorded using data loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: $\pm 0.53°C$, spectral detection range: 150–1200 nm), and lux values were converted to photosynthetically active radiation (PAR) using a conversion factor (1 μmol quanta m^{-2} s^{-1} = 52.0 lux) obtained through an inter-calibration with a PAR sensor (LI-COR LI-192SA underwater quantum sensor). Dissolved inorganic N (DIN, i.e., NH_4^+ = ammonium + NO_x = nitrogen oxides), dissolved inorganic phosphate (DIP), particulate (POC) and dissolved organic C (DOC), particulate N (PN) and chlorophyll *a* concentrations were monitored through in situ water sampling at 10 m water depth ($\sim$ 1 m above the benthos) and subsequent determination using standard analytical methods. For a detailed description of each method, please refer to Bednarz et al. (2015) and Cardini et al. (2015).

Sample collection

Once per season, fully developed seagrass ramets ($n = 8$) were collected at approximately 10 m water depth using SCUBA. We selected ramets of similar size (two shoots, approximately 16 leaves per ramet), excised the rhizome and collected the ramets complete of leaves, rhizome, and roots. Care was taken to minimize root damage. Samples were immediately transported back to the MSS and transferred

into an outdoor flow-through aquarium (800 L) supplied with seawater pumped directly from the reef at 10 m depth (exchange rate: 4000 L h^{-1}) to ensure in situ water temperature and nutrient concentrations. Light intensity in the aquarium was monitored with lux and PAR data loggers (see above) and adjusted with black netting to in situ values at 10 m water depth. Seagrass ramets were fixed to ceramic tiles with rubber bands while preventing tissue damage. All samples were maintained in the aquarium until the subsequent incubations for net primary productivity and N_2 fixation measurements took place over the following 2 d. The glass chambers (total volume = 1 L) were kept in the flow-through aquarium during all incubations described in the following section, ensuring in situ temperature and irradiance throughout the incubation periods. After the incubations, the surface area of each seagrass ramet was obtained by doubling their planar surface area, which was measured from planar photographs of all leaves, rhizomes, and roots of each ramet using Image J software (Schneider et al. 2012).

Quantification of primary productivity

O_2 fluxes as a proxy for primary productivity were quantified during two individual incubations. The first incubation was carried out ca. 2 h after sunset to measure dark respiration (R_D), while the second incubation started the following day at 12:00 h for net photosynthesis (P_N) determination. Seagrass ramets were incubated individually and additional chambers with unfiltered seawater (without seagrass, $n = 4$) or with ceramic tiles (without seagrass, $n = 4$) served as controls to measure background metabolism. Chambers were sealed with a spring-loaded glass lid and incubated under constant stirring (600 rpm) for 60–90 min. Dissolved O_2

concentrations were measured at the start and end of each incubation period using a salinity and temperature corrected O_2 optode sensor (MultiLine ® IDS 3430, WTW GmbH). O_2 fluxes were corrected for the mean O_2 difference of the seawater controls (ceramic tiles had no effects) and normalized to incubation time and surface area of the respective ramet. Finally, gross photosynthesis (P_G) was calculated subtracting R_D from P_N for each ramet.

Quantification of N_2 fixation

The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) as a proxy for N_2 fixation was measured in seagrass ramets using an adapted C_2H_2 reduction assay (Capone 1993; Wilson et al. 2012) during a full dark-light cycle incubation (24 h). Samples were incubated in the same chambers described above but with the presence of a headspace (20% of the total volume). Immediately prior to the start of the incubations, 10% of the seawater in the chamber was replaced with C_2H_2-saturated seawater. Chambers were then sealed gastight and 10% of the headspace was replaced with C_2H_2 gas via a needle injection port in the glass lid. Chambers were stirred (600 rpm) throughout the incubation period. Incubations started and ended just prior to sunset (approx. 17:00 h). Gas samples were collected from each chamber after 0, 4, 12, 16, and 24 h to capture the periods of dusk, night, dawn, and full daylight (except during spring when samples were taken only at 0, 4, and 24 h, and only the mean daily N_2 fixation rate was calculated). We selected these sampling times to capture periods of the day with different light conditions that are important in regulating nitrogenase activity associated with both benthic organisms (Lesser et al. 2007) and diazotrophic bacterioplankton (Hewson et al. 2007) in tropical reef environments. C_2H_4 concentrations in the gas samples were measured using a customized reducing compound photometer (RCP; Peak Laboratories, detection limit = 100 ppb), and C_2H_4 evolution rates were calculated according to Breitbarth et al. (2004). Daytime, nighttime and daily mean N_2 fixation rates (Nf_L, Nf_D, and Nf, respectively) were calculated correcting for the signal of unfiltered seawater controls ($n = 8$) and normalized to incubation time and surface area of the ramets. Three additional controls with (1) only 0.2 μm-filtered seawater (without seagrass, $n = 6$), (2) ceramic tile in unfiltered seawater (without seagrass, $n = 6$), and (3) seagrass ramets in unfiltered seawater, but with no C_2H_2 addition (natural C_2H_4 production, $n = 6$), were incubated as well and showed negligible C_2H_4 evolution.

Data extrapolation and analyses

To extrapolate the measured physiological rates to the entire seagrass meadow, fluxes were normalized to the number of leaves of each incubated ramet (and not to ramet surface area as above), and then multiplied by the mean leaves density of the meadow (Table 2). O_2 fluxes were converted into dissolved inorganic C fluxes using a theoretical photosynthetic (and respiratory) quotient of 1 mol O_2 produced

Table 1. Meadow and plant features of the *H. stipulacea* settlement in front of the Marine Science Station (Aqaba, Jordan). Values are means $\pm$ SD.

Feature	Value
Water depth range (m)	$\sim$5–30
Areal extent (m^2)	$\sim$2400
Shoot density (no. m^{-2})	1093 ± 311
Leaves density (no. m^{-2})	8747 ± 2487
Mean leaf length (mm)	50.5 ± 7.7
Mean leaf width (mm)	7.7 ± 1.2
Leaf area index (m^2 m^{-2})	2.94 ± 0.83

(consumed) per mol CO_2 fixed (released) (Duarte et al. 2010). Net Community Production (NCP) in units of mmol C m^{-2} meadow area d^{-1} was then calculated according to NCP = GPP - CR, where CR is the community respiration, calculated from R_D as the mmol C consumed m^{-2} meadow area over the 24 h, and GPP is the gross primary production calculated from P_G as the mmol C produced m^{-2} meadow area during daytime (approximated to 12 h of daylight). C_2H_4 evolution rates were converted into meadow biological N_2 fixation (BNF) using a theoretical ratio of 3 mol C_2H_4 produced per mol N_2 fixed (Welsh 2000), and are expressed in units of mmol N fixed m^{-2} meadow area d^{-1}. A C/N ratio of 23.7 was used (Wahbeh 1988) to estimate the seagrass meadow photo-metabolic N demand from its NCP and to subsequently calculate the % contribution of BNF to this demand.

Differences in each parameter were assessed using univariate distance-based permutational nonparametric analyses of variance (PERMANOVA) (Anderson 2001). Data were square root transformed, and analyses were based on Bray Curtis similarities. Absolute values were used in the case of R_D. A design with the single factor "Season" was used to test for differences (encompassing the combined effects of all environmental parameters) in P_N, R_D, Nf. A design with "Season" and "Time of day" as fixed factors was used to test for differences in Nf_L and Nf_D. Type I (sequential) sum of squares was used with 9999 unrestricted permutations of raw data, and pair-wise tests were carried out if significant differences occurred ($p < 0.05$). PERMANOVA tests were performed using the homonymous routine included in the software PRIMER 6+. The influence of individual environmental parameters on each physiological rate and the relationship between meadow NCP and BNF during each of the four seasons were further examined using linear regressions.

Results

Environmental settings

Meadow and plant features of the *H. stipulacea* settlement under study are reported in Fig. 1 and Table 1. All environmental parameters monitored at 10 m water depth showed

Table 2. Seasonal measurements of environmental parameters in the research area at 10 m water depth. Temperature and maximum PAR were measured continuously during each seasonal period while other parameters were measured weekly. Values are means (weekly averages, with 4 weeks in each seasonal period) $\pm$ SE. Feb, February; Apr, April; Sep, September; Nov, November.

Parameter	Winter (Feb)	Spring (Apr)	Summer (Sep)	Fall (Nov)
PAR (μmol m^{-2} s^{-1})	180 (15)	257 (9)	317 (17)	159 (18)
Temperature (°C)	23.0 (0.1)	22.8 (0.1)	27.5 (0.2)	25.2 (0.2)
DIN (μM)	1.03 (0.02)	1.02 (0.11)	0.20 (0.04)	0.43 (0.08)
DIP (μM)	0.11 (0.01)	0.10 (0.01)	0.04 (0.01)	0.04 (0.01)
DIN:DIP	9.59 (1.09)	10.21 (0.43)	5.31 (3.40)	11.25 (2.22)
POC (μM)	7.70 (0.40)	10.25 (0.72)	6.68 (1.07)	8.81 (0.98)
PN (μM)	0.93 (0.07)	1.27 (0.05)	0.78 (0.05)	0.87 (0.07)
POC:PN	8.32 (0.19)	8.07 (0.59)	8.91 (0.63)	10.11 (0.48)
DOC (μM)	76.62 (4.27)	71.95 (3.77)	84.71 (2.09)	80.81 (0.54)

marked seasonal variability over the study period, with the most pronounced differences occurring between a deep-water mixed season in winter and spring, and a stratified season in summer and fall (Silverman et al. 2007; Bednarz et al. 2015). The daily maximum irradiance was highest in summer and lowest in winter (Table 2). The in situ temperature ranged from 22.5°C to 27.5°C, remaining low throughout the deep-water mixed season, reaching a maximum in summer before decreasing again in fall (Table 2). Concentrations of inorganic nutrients were more than twice as high in the deep-water mixed season compared to the stratified season (Table 2). POC and PN peaked in spring during the seasonal plankton bloom, but POC : PN ratios exceeded the Redfield ratio (106 : 16) throughout the year, indicating a deficiency of N compared to Redfield proportions (Table 2). DOC concentrations were significantly higher during the stratified season than during the deep-water mixed season, and peaked in summer (Table 2).

Net photosynthesis and dark respiration of seagrass ramets

P_N of seagrass ramets ranged between 0.24 ± 0.02 and 0.44 ± 0.05 μmol O$_2$ cm^{-2} ramet area h^{-1}, and was significantly higher in spring and summer compared to winter and fall (Fig. 2a; Supporting Information Table 1). R_D ranged between -0.03 ± 0.001 and -0.08 ± 0.009 μmol O$_2$ cm^{-2} ramet area h^{-1} and was significantly higher in summer compared to the other seasons, with no statistical difference recorded between the winter and spring values (Fig. 2a; Supporting Information Table 1). Over the year, irradiance had a positive effect on both P_N and R_D, explaining most of the variation in the data, while no significant effects were found for the other environmental parameters (Table 3).

Daytime and nighttime N₂ fixation of seagrass ramets

Nf$_L$ ranged between 0.08 ± 0.01 and 0.89 ± 0.25 nmol C$_2$H$_4$ cm^{-2} ramet area h^{-1}, while Nf$_D$ ranged between 0.05 ± 0.01 and 5.37 ± 0.79 nmol C$_2$H$_4$ cm^{-2} ramet area h^{-1}. Both Nf$_L$ and Nf$_D$ reached the minimum values in winter and the maximum values in summer, with overall seasonal means (Nf) that were

between one and two orders of magnitude higher in summer compared to winter (Fig. 2b). Moreover, Nf$_D$ was approximately sixfold higher than Nf$_L$ in summer (Fig. 2b). Conversely, in the other seasons differences between Nf$_L$ and Nf$_D$ were insignificant (Fig. 2b; Supporting Information Table 2). Environmental parameters had a similar effect on both Nf$_L$ and Nf$_D$. In particular, irradiance, temperature and DOC exerted a positive control over N$_2$ fixation, while inorganic nutrients and particulate organic C and N had a negative effect (Table 3).

Meadow net community production and N₂ fixation

The results of the extrapolation of ramet-specific rates to the seagrass meadow community level are reported in Fig. 3 and Table 4. NCP had a parabolic response to environmental variations over the year, with an increase in production from winter to spring that corresponded to the increase in irradiance and a subsequent decrease again in fall, when light availabilities dropped again (Fig. 3a,b). Conversely, BNF showed a peak response in summer to the decrease in nutrient concentrations and simultaneous increase in temperature (Fig. 3a,c), with the resulting input of N increasing from 0.06 in winter to 4.60 mmol N m^{-2} d^{-1} in summer (i.e., an increase by two orders of magnitude). In summer, in contrast to the other seasons, there was a significant positive linear relationship between NCP and BNF (Table 5), with the latter accounting for ca. 20% of the photo-metabolic N demand of the meadow (Table 4).

Discussion

Here, we demonstrate the pivotal role of epiphytic N$_2$ fixation in providing N to the plant when the water column is most oligotrophic. This trait in turn may help the plant holobiont to cope with environmental variations typical of temperate seas, increasing its ecological resilience and invasiveness.

Diel and seasonal patterns in primary productivity and N₂ fixation

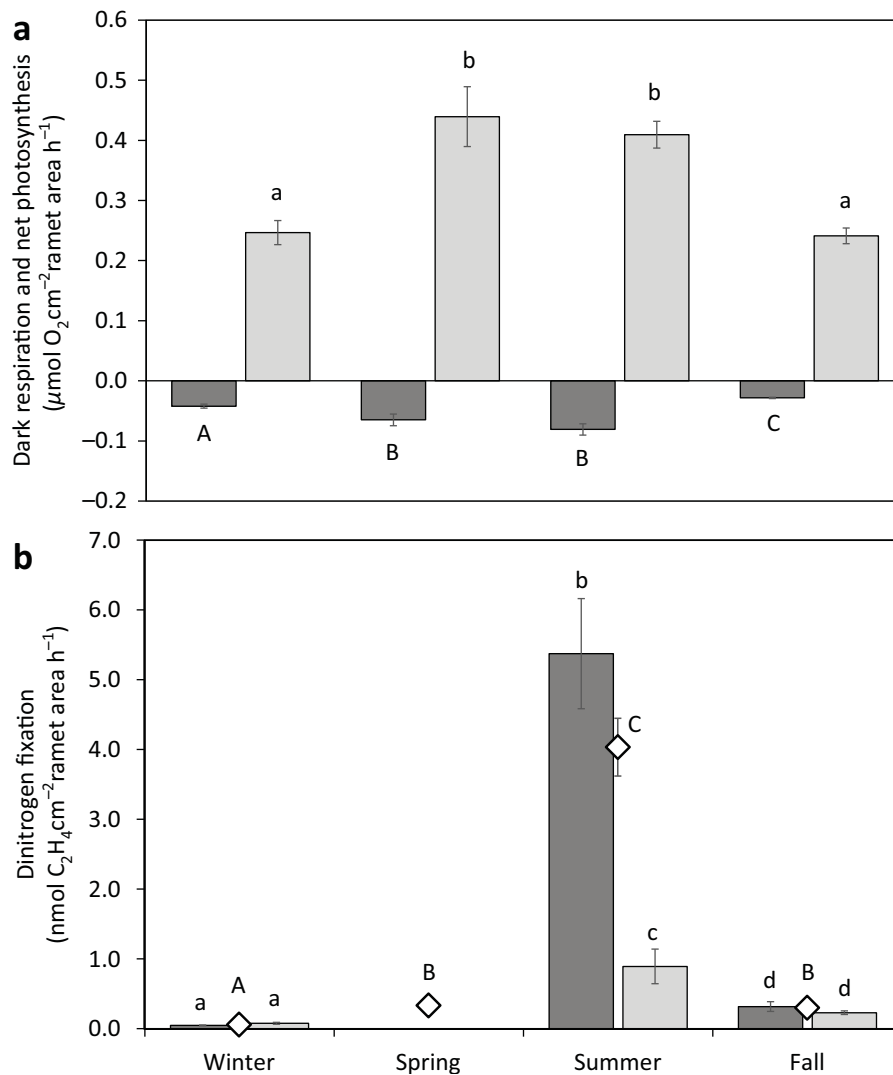*H. stipulacea* showed a diel net autotrophic metabolism, with P_N and R_D rates increasing in spring and summer

Fig. 2. (**a**) Dark respiration (dark gray), net photosynthesis (light gray), and (**b**) N_2 fixation by experimental *H. stipulacea* ramets in the four seasonal periods. In (**b**), nighttime (dark gray) and daytime (light gray) N_2 fixation rates are shown. Diamonds represent mean daily N_2 fixation rates. Significant pairwise comparisons for the factor "Season" or for the interaction term "Season" $\times$ "Time of day" ($p < 0.05$, PERMANOVA) are indicated as different letters. The use of small and capital letters indicates different analyses.

Table 3. Linear regression analyses (presented as R^2 values) of the influence of key environmental parameters on net photosynthesis (P_N), dark respiration (R_D) and nighttime and daytime N_2 fixation (Nf_D and Nf_L, respectively) of seagrass ramets. Bold values indicate a significant positive linear relationship and italicized values indicate a significant negative linear relationship. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant.

Variable	Irradiance	Temperature	DIN	DIP	POC	PN	DOC
P_N	**0.444*****	0.025ns	0.001ns	0.001ns	0.019ns	0.114ns	0.014ns
R_D	**0.500*****	0.093ns	0.030ns	0.002ns	0.037ns	0.004ns	0.012ns
Nf_L	**0.455*****	**0.397*****	*0.315****	*0.198**	*0.260**	*0.444****	**0.403*****
Nf_D	**0.804*****	**0.609*****	*0.446****	*0.243**	*0.553****	*0.719****	**0.620*****

compared to the other seasons, corresponding to the highest irradiance levels at the study site. This was confirmed by our regression analyses. The fast changes in metabolic activity of

H. stipulacea in response to changing irradiances are due to its high photo-physiological plasticity which allows it to occupy seafloor areas along a wide range of water depths
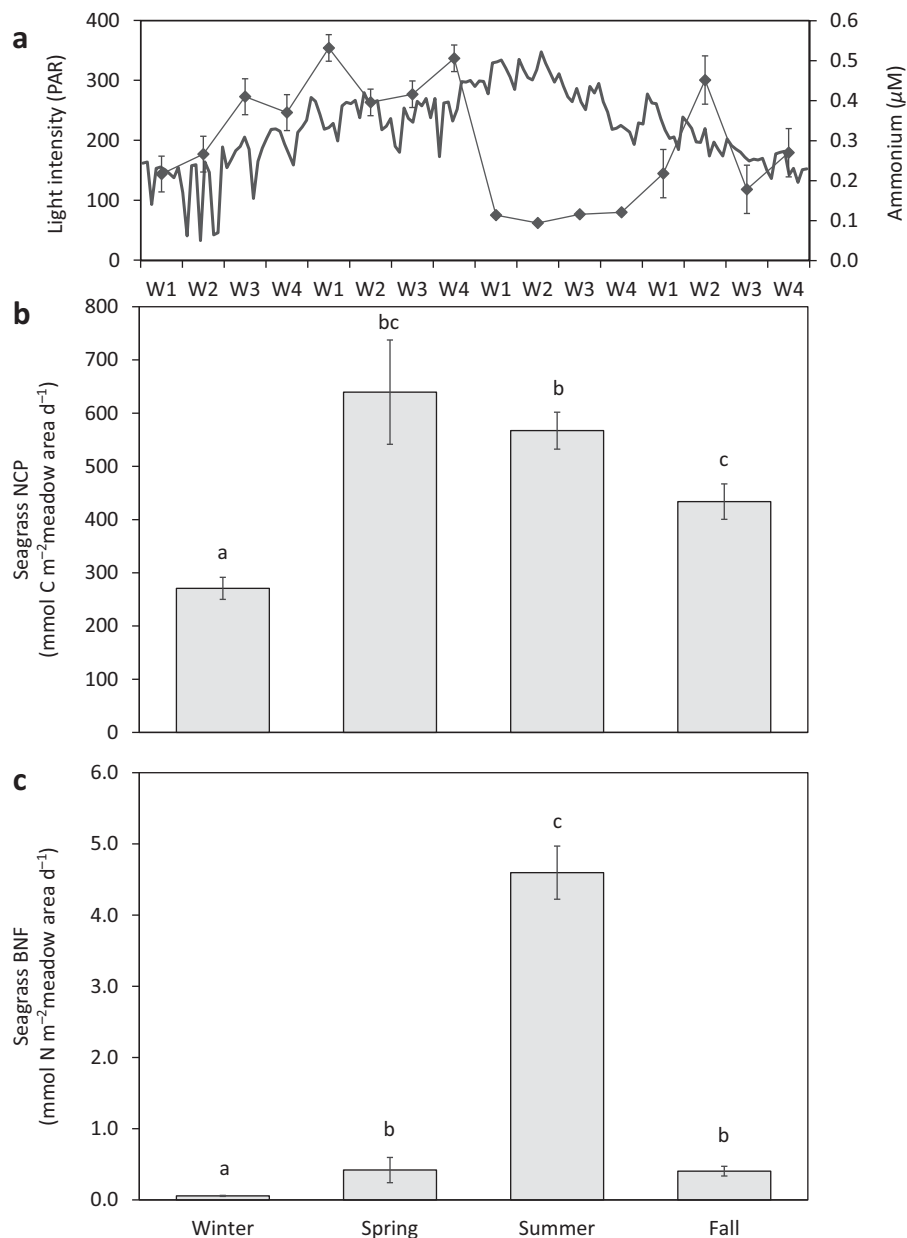
Fig. 3. (**a**) Daily maximum PAR and NH_4^+ concentrations (weekly averages, with 4 weeks in each seasonal period) as representative environmental factors at the study site (10 m water depth); (**b**) *H. stipulacea* meadow net community primary production (NCP) and (**c**) biological N_2 fixation (BNF) in the four seasonal periods. Significant pairwise comparisons for the factor "Season" ($p < 0.05$, PERMANOVA) are indicated as different letters.

Table 4. Biological N_2 fixation (BNF), gross primary production (GPP), community respiration (CR), net community production (NCP) in mmol (C or N) m^{-2} meadow area d^{-1}, and % contribution of BNF to NCP in the different seasons (minimum and maximum values are shown in parentheses).

	BNF	GPP	CR	NCP	% contribution
Winter	0.06 (0.03–0.09)	384 (271–474)	113 (80–147)	271 (188–347)	0.5 (0.3–0.9)
Spring	0.42 (0.11–1.61)	866 (513–1483)	226 (124–477)	640 (383–1097)	1.6 (0.3–5.2)
Summer	4.60 (2.99–6.16)	856 (642–1181)	289 (141–505)	567 (440–729)	19.3 (14.9–24.2)
Fall	0.40 (0.18–0.81)	549 (432–758)	115 (90–152)	434 (327–632)	2.3 (1.0–4.6)

Table 5. Linear regression analysis (presented as R^2 values) between seagrass meadow net community production (NCP) and meadow N_2 fixation (BNF) during each of the four seasonal periods. Bold values indicate a significant positive linear relationship. *$p < 0.05$, ns, not significant.

Season	Regression
Winter	0.034[ns]
Spring	0.035[ns]
Summer	**0.532***
Fall	0.015[ns]

(Runcie et al. 2009; Sharon et al. 2009; Rotini et al. 2017). This was also noted by Wahbeh for *H. stipulacea* from the Gulf of Aqaba in comparison to other species of the genus *Halophila* inhabiting the Gulf (Wahbeh 1983). Interestingly, there was no correlation of any other environmental parameter with P_N and R_D rates of *H. stipulacea*, indicating that this species is able to quickly capitalize on higher light availabilities even when other important factors (e.g., nutrient availability) change unfavorably.

Conversely, N_2 fixation associated with the seagrass ramets was highly variable both on a diel and on a seasonal basis. There was a general increase of approximately 1–2 orders of magnitude in N_2 fixation from winter to summer and a following decrease in fall, irrespective of day or night. In addition, the high summer rates were mainly due to Nf_D that were 6-times higher compared to Nf_L strongly indicating a potential role of heterotrophic bacteria attached to the seagrass leaves, roots, and rhizomes. In fact, Wahbeh and Mahasneh (1984) found that leaves, roots and rhizomes of *H. stipulacea* and other Halophila species from the Gulf are particularly dominated by heterotrophic bacteria (including heterotrophic diazotrophs) during summer.

N_2 fixation (during both day and night) showed positive and significant correlations with temperature, irradiance, and DOC. This is in line with other observations from the photic zone of the northern Gulf of Aqaba indicating that a shift occurs in the diazotrophic plankton community from phototrophic and heterotrophic populations in winter (including the cyanobacteria *Trichodesmium*) to predominantly heterotrophic diazotrophs in summer (Rahav et al. 2015). Net DOC fluxes in seagrass communities increase positively with water temperature and irradiance (Barrón et al. 2014), and higher irradiances and temperature combined with high photosynthesis rates in summer interact in determining higher DOC release by benthic algae (Haas et al. 2010; Mueller et al. 2016). At our study site, DOC concentrations increased in summer in the water directly above the benthos. Heterotrophic diazotrophs are C limited in summer in the Gulf of Aqaba (Rahav et al. 2015), and they may thus greatly benefit from DOC released by the seagrass and the associated epibionts. Higher DOC release rates by the

seagrass during summer may not only influence planktonic diazotroph, but also stimulate settlement and accumulation of bacteria (including diazotrophs) on the seagrass phyllosphere. Indeed, a recent study on *P. oceanica* from the Mediterranean Sea found highest epiphyte accumulation on the phyllosphere during summer that correlated positively with increased N_2 fixation rates (Agawin et al. 2016). Enhanced settlement of nutrient-providing bacteria during times of nutrient shortage seems to be an effective strategy to maintain a high productivity during the oligotrophic summer. Besides C, higher seagrass photosynthesis and respiration rates in summer may also facilitate higher absorption of manganese and iron (Wahbeh 1984b), which can otherwise be elements that also limit N_2 fixation.

Interestingly, two other studies used both in situ and laboratory experiments to study the dynamics of N_2 fixation in different micro-niches of *H. stipulacea* from the Gulf of Aqaba (Pereg et al. 1994; Pereg-Gerk et al. 2002), although from a different location. Both these studies concluded that N_2 fixation is light-dependent in the *H. stipulacea* meadow and attributed the activity to cyanobacteria and Rhodospirillacean bacteria occupying the aerobic and anaerobic niches of the phyllosphere, respectively. Although at a first look these results may be contradicting our findings, these studies were carried out in winter (December 1991–May 1992), when deep water mixing occurs in the Gulf of Aqaba and the conditions favor phototrophic diazotrophs (Rahav et al. 2015). Albeit rates were low and differences not significant, we found average N_2 fixation in winter to be higher in the daylight. This supports the hypothesis of a community of seagrass-associated diazotrophic epiphytes driven by seasonality and dominated by phototrophs in winter and spring and by heterotrophs in summer and fall. We also found DIN, DIP, POC, and PN to negatively and significantly correlate with N_2 fixation (Table 3). Although the presence of readily available reactive N (dissolved or particulate) in the environment is known to limit N_2 fixation, an inhibitory effect of DIP or POC seems contradicting current knowledge (Carpenter and Capone 2008). However, the outcome of these regression analyses is likely driven by a confounding effect of simultaneous fluctuations over the year for POC and PN, and DIN and DIP in the Gulf of Aqaba, which are directly affected by seasonal forcing (i.e., deepwater mixing vs. stratification). In support of our interpretation, the water column at our study site exhibited POC : PN and DIN : DIP ratios that were respectively higher and lower than Redfield (C : N : P = 106 : 16 : 1) during all seasons (Table 3), indicating N rather than P as the nutrient limiting productivity (and thus also exerting control on N_2 fixation) at our study site.

C and N budget and contribution of N_2 fixation to primary productivity

The BNF, GPP, CR, and NCP reported here for a *H. stipulacea* meadow fall in the range of those summarized from

previous studies looking at these processes in other seagrass species from the tropics (*see* Cardini et al. 2014; Welsh 2000; Duarte et al. 2010 for reviews), despite the large variations of metabolic rates (Duarte et al. 2010). CR data compare well also with similar extrapolations at the ecosystem level presented in Cardini et al. (2016) for a coral-dominated community adjacent to the *H. stipulacea* meadow for which results are here presented. However, GPP and NCP were significantly higher for the seagrass meadow compared to the coral reef, particularly in spring and summer. This is not uncommon, and similar results have been reported from other sites, where seagrasses contributed most of the primary production to the overall ecosystem (e.g., Naumann et al. 2013). *H. stipulacea* also constantly showed high NCP rates and GPP : R ratios well above 1, in line with previous observations that demonstrated that seagrass meadows tend to be net autotrophic and therefore act as an important CO_2 sink in the marine realm (Duarte et al. 2010). BNF was higher in the seagrass compared to the adjacent coral-dominated community, with fourfold higher rates in summer (Cardini et al. 2016), supporting other literature that ranks seagrass ecosystems among the major contributors to benthic N_2 fixation, before coral reefs (*see* Capone and Carpenter 1982; Cardini et al. 2014). N_2 fixation rates per cm^{-2} surface area on *H. stipulacea* are lower than the rates reported for the non-vegetated silicate sand at the same study site (Bednarz et al. 2015). However, the reverse is true when we take into account the tri-dimensional surface area of the seagrass canopy to extrapolate the flux to a flat square meter of seafloor area (i.e., BNF, in mmol N m^{-2} d^{-1}). This extrapolation results in a yearly average of 1.37 ± 0.16 mmol N m^{-2} d^{-1} from the seagrass, while only 0.24 ± 0.02 mmol N m^{-2} d^{-1} originated from the non-vegetated silicate sand (Bednarz et al. 2015), i.e., approximately 6 times lower. This difference would likely increase significantly, if we would also consider the contribution of heterotrophic N_2 fixation within the vegetated sediments (i.e., the contribution of the rhizosphere, not quantified here), which is favored by root exudation and radial oxygen loss and can be high particularly in tropical and subtropical environments (Patriquin and Knowles 1972).

The contribution of BNF to NCP in the seagrass was important only in summer, when we also detected a significant correlation between the two processes. This is remarkable, because it suggests that BNF strongly supports primary production by *H. stipulacea* when other nutrient sources are lacking. The low tissue N content (high C : N ratio) of this species in the Gulf of Aqaba (Wahbeh 1988; Schwarz and Hellblom 2002) suggests low N availability, which need to be accounted for when determining constraints on growth of *H. stipulacea* in the study region. Interestingly, the maximum production of new leaves in the meadow under study has been reported for November (Wahbeh 1984*a*), which coincided with maximum N content of leaves (Wahbeh 1988). This may support the hypothesis that diazotrophs provide the plant with bioavailable N in summer that is then stored and used in fall for production of new leaves and growth of the meadow. However, a sampling design with higher temporal resolution, particularly during the seagrass' actively growing season, and the use of additional methods (e.g., stable isotope probing and Nano-scale Secondary Ion Mass Spectrometry) would be necessary to conclusively test this hypothesis.

Ecological implications

The NCP reported here indicate that *H. stipulacea* has very high productivity and growth potential which is likely an important trait in determining the invasiveness of this seagrass. This was also confirmed by a recent study that showed that temperature is not a limiting factor for the expansion of this species, which was able to survive, photosynthesize, and grow within a broad range of temperatures (10–30°C) (Georgiou et al. 2016). Our study further indicates that *H. stipulacea* is a very suitable substrate for diazotrophs that actively fix N and thus provide an additional N source to be used for plant production and growth. Recently, high N_2 fixation rates and a diverse community of diazotrophs have been reported for roots of the endemic Mediterranean seagrass *P. oceanica* (Agawin et al. 2016; Garcias-Bonet et al. 2016; Lehnen et al. 2016), suggesting that N_2 fixation may support growth also in this primary producer. While this is likely, *H. stipulacea* possesses characteristics that are exceptional compared with other tropical and temperate seagrass species, such as a high affinity for NH_4^+ uptake, a similar uptake capacity and efficiency of leaves and roots and a high invasiveness potential in sites under eutrophic conditions (Alexandre et al. 2014; van Tussenbroek et al. 2016). At comparable ambient NH_4^+ concentrations, NH_4^+ uptake rates of *P. oceanica* leaves are roughly 3 orders of magnitude lower than those reported for *H. stipulacea* (Apostolaki et al. 2012; Alexandre et al. 2014). Average N_2 fixation rates measured here in *H. stipulacea* during summer were ~ 4 times higher (64.4 ± 5.2 mg N m^{-2} d^{-1}) as compared to maximum areal rates reported for *P. oceanica* (15.2 mg N m^{-2} d^{-1}) (Agawin et al. 2016) suggesting an ecological advantage for *H. stipulacea*. Further, eutrophic conditions were shown to favor *H. stipulacea* expansion (van Tussenbroek et al. 2016), while they cause epiphyte overgrowth that further hinders N uptake by *P. oceanica* leaves (Apostolaki et al. 2012). To conclude, *H. stipulacea* is a seagrass species with a high physiological plasticity that can be advantageous in the colonization of new habitats. Quickly capitalizing on the products of N_2 fixation by associated diazotrophs may further allow this species to spread in areas with variable N concentrations, such as the coastal waters of the Mediterranean Sea or of the Caribbean, and to outcompete endemic species such as *P. oceanica*.

Author Contribution

UC and CW designed the experiments and coordinated collection of data and lab work. UC carried out the statistical

analyses and wrote the manuscript with support from CW, who coordinated the project. NvH and VNB contributed in designing the experiments and participated in the collection of the data and the lab work. MMDA advised on the field activities. All authors contributed to and approved the final manuscript.

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Acknowledgments

Conflict of Interest

None declared.