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Seasonality affects dinitrogen fixation associated with two common macroalgae from a coral reef in the northern Red Sea

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ABSTRACT: Nitrogen (N) is often a limiting nutrient for primary production in coral reef ecosystems. In this context, dinitrogen (N_2)-fixing prokaryotes (diazotrophs) associated with benthic primary producers can relieve N limitation. Macroalgae are key reef players that are generally able to rapidly uptake dissolved inorganic nutrients. They may thus particularly benefit from the activity of associated diazotrophs. With this rationale, this study investigated N_2 fixation activity and net primary production associated with 2 dominant coral reef macroalgae (the green algal genus *Caulerpa* and the brown algal genus *Lobophora*) during all 4 seasons in a fringing northern Red Sea reef using the acetylene using the acetylene reduction assay and oxygen production and consumption measurements. Both macroalgae exhibited associated N_2 fixation activity during all seasons with lowest activity in winter and significantly higher activity (1 and 2 orders of magnitude increase for *Lobophora* and *Caulerpa*, respectively) during the nutrient-depleted summer, while net primary production for both macroalgae remained relatively constant over all 4 seasons. Primary production rates of the macroalgae were comparable to corals from the same area on a yearly average. Conversely, average N_2 fixation rates of both macroalgae were approximately 5-fold higher than rates reported for hard corals that were incubated in parallel experiments. These results indicate that macroalgae can capitalize on higher inputs of N from epibiotic diazotrophs, which in turn could prove an ecological advantage when competing for space with corals.

KEY WORDS: Gulf of Aqaba · Acetylene reduction · Primary production · Macroalgae · Dinitrogen fixation

INTRODUCTION

Coral reefs are systems that exhibit notably high primary production despite being surrounded by oligotrophic waters (Odum & Odum 1955). Due to the scarcity of bioavailable nutrients, benthic organisms such as hard and soft corals have evolved an effective mutualistic symbiosis with single-celled dinoflagellates of the genus *Symbiodinium* (also known as zooxanthellae) for maintaining efficient uptake, recycling and conservation of (in)organic carbon (C), phosphorus (P) and nitrogen (N) (Davy et al. 2012, Kopp et al. 2013, Ferrier-Pagès et al. 2016). In this biogeochemical cycling of nutrients, N is often considered the limiting factor that controls primary productivity (i.e. the fixation of inorganic C through photosynthesis), and is therefore an essential macronutrient for zooxanthellae (Falkowski 1997, Wang & Douglas 1999). Uptake of

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ammonium by the zooxanthellae as a source of inorganic N is preferred over uptake of other forms such as nitrate (Ezzat et al. 2015). Bioavailable N is lost when nitrifying and denitrifying bacteria act together to transform ammonium into dinitrogen (N_2), which most organisms cannot use. However, N_2-fixing prokaryotes, or diazotrophs, are able to restock the bioavailable N pool by converting N_2 into ammonium. Zooxanthellate corals appear to have evolved characteristic associations with diazotrophs (Lema et al. 2012, 2014, Bednarz et al. 2015a), and recent research indicates that N_2 fixation can provide hard corals with a significant portion of their daily N requirements (Cardini et al. 2015, Benavides et al. 2016). Thus, the association with diazotrophs may be key to their success in oligotrophic waters.

Besides corals, N_2 fixation activity has been measured for other benthic organisms that represent important functional groups on coral reefs (see Cardini et al. 2014 for a review), namely macroalgae (e.g. Capone et al. 1977). Here, diazotrophs inhabit the macroalgae epibiotically (e.g. Fong et al. 2006, Lachnit et al. 2011), often in high abundances (Maximilien et al. 1998), and may play an important ecological role in (a)biotic interactions with the macroalgal host (Wahl 2008, Wahl et al. 2012). These macroalgae–diazotroph interactions could provide the macroalgae with otherwise unavailable nutrients and may be essential for their competitive success. Until recently, N_2 fixation in coral reefs did not receive much attention, and data on macroalgae–diazotroph interactions is particularly lacking. To our knowledge only a few studies on macroalgae-associated N_2 fixation activity are available (e.g. Carpenter 1972, Head & Carpenter 1975, Capone et al. 1977, Penhale & Capone 1981, Hamersley et al. 2015) and some only report N_2 fixation rates from a single isolated species of diazotroph (Carpenter 1972, Head & Carpenter 1975). These studies reported N_2 fixation rates for the genera *Sargassum*, *Codium*, *Macrocystis*, *Laurencia*, *Microdictyon*, *Dictyota*, *Padina*, and *Halimeda*. Of these, the latter 5 were collected from coral reefs.

Anthropogenic stressors will continue to act upon coral reef systems in the future (Hoegh-Guldberg et al. 2007), and may well lead to a loss of coral cover (or recruitment) and/or an increase in (macro)algal cover (Lapointe 1997, Hoegh-Guldberg et al. 2007, Albright et al. 2010). This can cause a progressive shift from coral domination towards (macro)algae domination in what is called a phase shift (Done 1992). In this context, recent studies suggest that ocean acidification and increased sea surface temperatures as well as eutrophication can alter diazotrophic communities associated with hard corals (Santos et al. 2014, Rädecker et al. 2015) as well as their N_2 fixation activity (Rädecker et al. 2014, Cardini et al. 2016a). While the coral symbiosis is highly adapted to a low-nutrient regime, and zooxanthellae population densities are effectively controlled by the host by limiting nutrient availability to the algae (Falkowski et al. 1993), macroalgae are usually fast-growing organisms that quickly capitalize on pulses of dissolved nutrients that are otherwise rarely available (den Haan et al. 2016). In this context, N_2 fixation activity may give macroalgae an additional competitive advantage over corals, especially in a warming ocean (Rädecker et al. 2015) and/or in the absence of herbivores due to overfishing (Bellwood et al. 2006). Further studies examining the environmental factors that control the activity of macroalgae-associated diazotrophs are thus important if we want to understand the potential mechanisms underlying coral–algal phase shifts.

N_2 fixation is dependent upon several factors such as light, oxygen (O_2) concentrations, temperature, water flow, and availability of nutrients (Williams & Carpenter 1998, Sohm et al. 2011, Knapp 2012, Cardini et al. 2014). Furthermore, seasonal differences in N_2 fixation have been observed in hard corals (Cardini et al. 2015), soft corals (Bednarz et al. 2015a), sponges (Rix et al. 2015), turf algae (Rix et al. 2015), coral rock (Rix et al. 2015), and sediments (Bednarz et al. 2015b), but not macroalgae. Bednarz et al. (2015a), Cardini et al. (2015) and Rix et al. (2015) also suggested that N_2 fixation rates are correlated with productivity of the associated organism, which was especially evident during summer when light intensity and water temperature were highest and nutrient availability lowest. Moreover, Rix et al. (2015) found exceptionally high N_2 fixation associated with turf algae compared to values measured in other benthic organisms such as hard corals, likely due to a frequent association of turf algae with cyanobacteria (e.g. Cetz-Navarro et al. 2015). A high proportion of this fixed nitrogen is translocated to the eukaryotic part of the turf algae assemblage (e.g. Rhodophyta, Chlorophyta, and Phaeophyceae) and may thus provide the turf algae with a competitive advantage over other benthic organisms such as hard corals.

The present study extends the current literature by investigating (1) N_2 fixation and primary production associated with 2 common reef macroalgal genera, i.e. *Caulerpa* and *Lobophora*, (2) whether N_2 fixation and primary production are linked, and (3) which environmental factors drive macroalgae primary pro-

duction and associated N_2 fixation. Finally, we explore (4) how macroalgae-associated N_2 fixation and primary production compare to other key benthic reef organisms and (5) what the biogeochemical implications for coral–algal phase shifts could be. N_2 fixation and primary production rates, for both macroalgae and a set of key environmental parameters, were measured during all 4 seasons of the year 2013 in a northern Red Sea reef. The same methodology (acetylene reduction assay and O_2 measurements, i.e. production and consumption) and normalization parameters (species surface area) were used as in parallel incubation experiments that targeted different benthic reef organisms (e.g. see Rix et al. 2015, Bednarz et al. 2015a) to facilitate comparison of results.

MATERIALS AND METHODS

Study site and environmental monitoring

This study was conducted in 2013 at a fringing coral reef located within a marine reserve in front of the Marine Science Station (MSS) at the northern Gulf of Aqaba, Jordan (29° 27′ N, 34° 58′ E). The area is characterized by strong regional seasonality reflected by substantial variability of environmental key parameters throughout the year (Silverman et al. 2007, Carlson et al. 2014). In order to examine the effect of seasonality on macroalgae-associated N_2 fixation and primary production, all experiments described below were repeated over 4 seasonal periods in 2013: February (winter), April (spring), September (summer), and November (autumn).

Environmental parameters were continuously recorded at the sampling location at 10 m water depth over the course of the entire study period. This included daily measurements of *in situ* water temperature and light intensity using data loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: ±0.53°C, spectral detection range: 150 to 1200 nm) and a quantum sensor (LI-COR LI-192SA), and weekly collection and processing of seawater samples to quantify inorganic nutrients (dissolved inorganic nitrogen [DIN = ammonium + nitrate + nitrite] and phosphate [DIP]; fluorometrically for ammonium or photometrically for the remaining nutrients), particulate nitrogen (PN), particulate organic carbon (POC), and chlorophyll *a* (chl *a*; fluorometrically) concentrations. A detailed description of the sample and data analysis can be found in Bednarz et al. (2015b) or Rix et al. (2015).

Algae collection and maintenance

Individual fragments (n = 8) of 2 macroalgal genera, *Caulerpa* sp. and *Lobophora* sp. (herein referred to as *Caulerpa* and *Lobophora*, respectively), were collected during each season from the reef slope at 10 m water depth using SCUBA. *Caulerpa* fragments were carefully retrieved with their holdfasts from the sediment, while *Lobophora* leaves were carefully removed from their anchoring rock. All macroalgae were transferred to an outdoor 800 l flow-through aquarium supplied with seawater pumped directly from the reef at 10 m water depth (exchange rate: 4000 l h^{-1}), thereby providing *in situ* water temperature and nutrient levels. Layers of netting were positioned above the tank to adjust light levels to those measured *in situ* at 10 m water depth. The algae were allowed to acclimate for approximately 24 h before the incubations described below were carried out in the aquarium under the same environmental conditions.

Quantification of N_2 fixation and primary production

A detailed description of the chamber incubation procedure to quantify algae-associated N_2 fixation and primary production rates, as net photosynthesis (P_{net}) and dark respiration (R_{dark}), can be found in Bednarz et al. (2015a). Briefly, N_2 fixation was quantified by an adapted acetylene (C_2H_2) reduction technique (Capone 1993, Wilson et al. 2012). Macroalgae were incubated under constant stirring (600 rpm) over a full dark–light cycle (24 h) under maximum seasonal irradiance (see Table 1) in 1 l chambers with the seawater (0.8 l) and headspace (0.2 l) being 10% C_2H_2-enriched. Gas samples were drawn after 0, 4, 12, 16 and 24 h, and analyzed for ethylene (C_2H_4) concentration using a customized reducing compound photometer (Peak Laboratories, detection limit 100 ppb). P_{net} rates were quantified via O_2 production measurements over 60 to 90 min between 12:00 and 14:00 h, while R_{dark} incubations were conducted 1 to 2 h after sunset in complete darkness for 90 to 120 min using a conductivity- and temperature-corrected O_2 optode sensor (MultiLine® IDS 3430, WTW, accuracy: ±0.5% of measured value). Macroalgae were incubated under identical conditions as for N_2 fixation in individual 1000 ml closed cell respirometric glass chambers. P_{net} and R_{dark} were calculated by subtracting the initial O_2 concentration from the end concentration, and C_2H_4 evolution in each incubation chamber was calculated according to Breitbarth et al. (2004). C_2H_4 and O_2

measurements in each incubation chamber were control-corrected (unfiltered seawater) and normalized to incubation time and macroalgal surface area (see van Hoytema et al. 2016).

Statistical analysis

As not all assumptions for standard tests (e.g. ANOVA) were met, the data were analyzed using the non-parametric permutational multivariate analysis of variance (PERMANOVA). To test for differences in parameters (N_2 fixation, P_{net} and R_{dark}) between macroalgae genera and seasons, 2-factor PERMANOVAs were performed, based on Bray Curtis similarities of normalized and square-root transformed data. Therefore, Type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations), and pairwise-tests were carried out when significant differences occurred. Statistical analyses were carried out using Primer-E version 6 software (Clarke & Gorley 2006) with the PERMANOVA+ add on (Anderson 2001).

Correlations between N_2 fixation rates, P_{net} and R_{dark} rates per season and across all seasons followed by correlation analyses with environmental water parameters across seasons were determined via linear regression using Sigmaplot 12 (Systat software). Unless specified otherwise, significance level was set at $\alpha = 0.05$.

Table 1. Summary of key environmental water parameters monitored at 10 m water depth during 4 seasons. PAR: photosynthetically active radiation; DIN: dissolved inorganic nitrogen; DIP: dissolved inorganic phosphate; POM (POC + PN): particulate organic matter; POC: particulate organic carbon; PN: particulate nitrogen. Values are represented as means (n = 4) with SE in parentheses (from Bed-narz et al. 2015b)

Environmental variable	Winter	Spring	Summer	Autumn
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)
Ammonium (µM)	0.32 (0.04)	0.46 (0.03)	0.14 (0.03)	0.28 (0.06)
Nitrate (µM)	0.34 (0.03)	0.44 (0.04)	0.04 (0.01)	0.13 (0.05)
Nitrite (µM)	0.37 (0.06)	0.12 (0.04)	0.02 (0.01)	0.02 (0.01)
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)
POM (µM)	7.18 (0.70)	11.52 (1.48)	8.92 (1.23)	9.68 (0.49)
POC:PN	7.34 (0.57)	8.18 (0.59)	8.34 (0.44)	10.20 (0.51)
Chl *a* (µg l^{-1})	0.21 (0.01)	0.22 (0.02)	0.10 (0.01)	0.19 (0.02)

RESULTS

Seasonal variations of key environmental factors

All monitored environmental parameters exhibited a strong seasonal pattern with maximum light intensity and maximum water temperature during summer, while inorganic nutrients (i.e. DIN and DIP) and chl *a* concentration were lowest during summer (Table 1). Conversely, winter and spring displayed the most distinct environmental parameters compared to summer, followed by autumn (Table 1).

N_2 fixation activity associated with *Lobophora* and *Caulerpa*

Both macroalgae exhibited associated N_2 fixation during all 4 seasons indicated by high C_2H_4 evolution rates in algae-containing incubation chambers, while rates in the seawater controls were negligible. Macroalgae-associated N_2 fixation activity, expressed per algal surface area and averaged across the 4 seasons, resulted in similar values, i.e. 0.89 ± 0.19 and 1.07 ± 0.24 nmol C_2H_4 cm^{-2} h^{-1} for *Lobophora* and *Caulerpa*, respectively. In a seasonal comparison, *Caulerpa* revealed maximum N_2 fixation rates during spring and summer and lowest rates in winter, followed by autumn (Fig. 1a). In contrast, *Lobophora* showed significantly increased N_2 fixation rates during summer ($p < 0.001$), while the lowest rates were measured in winter followed by spring and autumn (Fig. 1a).

Primary production of *Lobophora* and *Caulerpa*

P_{net} and R_{dark} differed significantly between the 2 investigated macroalgae genera ($p < 0.001$). *Caulerpa* displayed higher rates than *Lobophora*, averaging 0.900 ± 0.059 and 0.300 ± 0.015 µmol O_2 cm^{-2} h^{-1} for P_{net} and 0.096 ± 0.011 and 0.067 ± 0.015 µmol O_2 cm^{-2} h^{-1} for R_{dark}, respectively, across all seasons. P_{net} was similar in winter, spring, and summer for both macroalgae, but decreased significantly for both genera from summer to autumn (Fig. 1b). P_{net} was similar for autumn and winter in *Caulerpa*, but similar for autumn and spring in *Lobophora* (Fig. 1b). R_{dark} was significantly higher in *Caulerpa* compared to *Lobophora* in all seasons except autumn (Fig. 1c) but followed roughly the same fluctuating pattern throughout the year for both macroalgae.

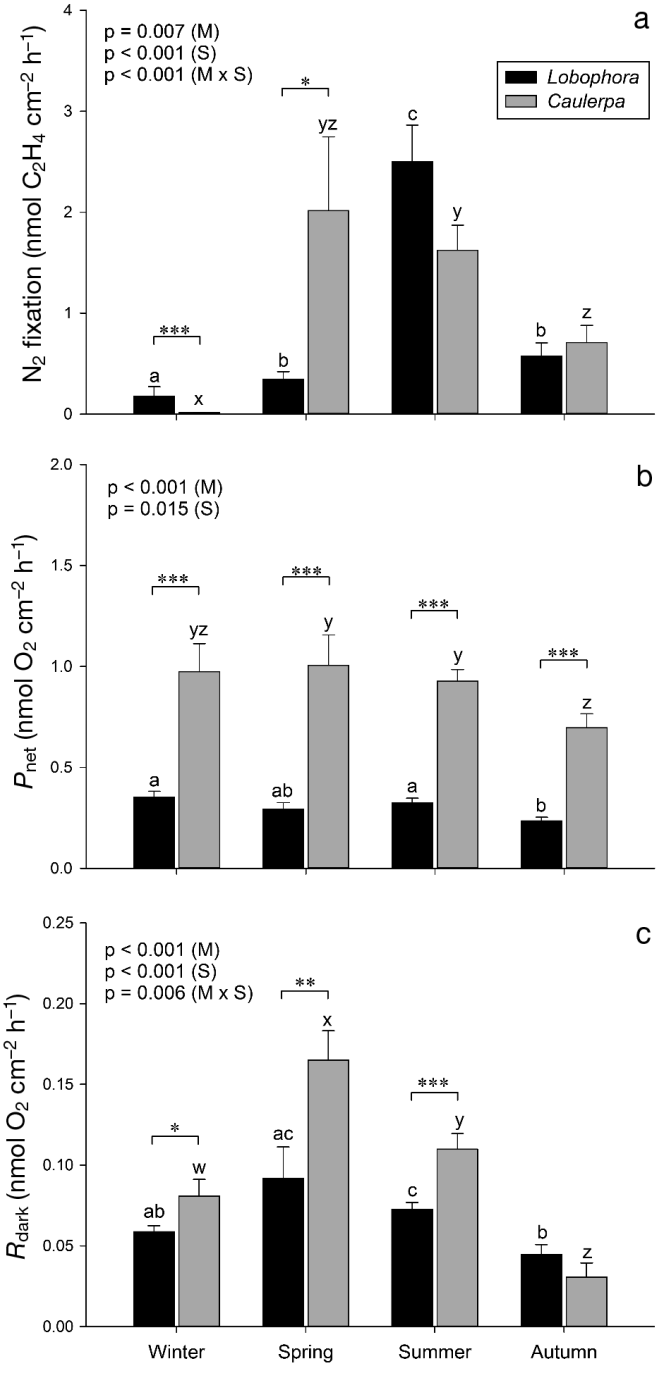

Fig. 1. (a) N_2 fixation, (b) net photosynthesis (P_{net}), and (c) dark respiration (R_{dark}) associated with the 2 macroalgae (*Caulerpa* and *Lobophora*) measured during 4 different seasons (winter, spring, summer, autumn). Values are given as mean ± SE (n = 8). The significant factor (M = macroalgae, S = season, M × S = interaction) is displayed for each parameter. Significant differences between the macroalgae during each season are indicated by *p < 0.05, **p < 0.01, ***p < 0.001. Different letters indicate significant differences within each macroalga per parameter between the seasons (*Lobophora*: a, b, c; *Caulerpa*: w, x, y, z), based on pair-wise PERMANOVA analysis

Relationships of metabolic rates and environmental factors

Linear regression analyses revealed a negative relationship between N_2 fixation and P_{net} for *Lobophora* during autumn ($F = 23.85$, $r^2 = 0.799$, $p = 0.003$) (Fig. 2d), while a positive relationship was found in the same season for *Caulerpa* ($F = 8.25$, $r^2 = 0.579$, $p = 0.03$) (Fig. 2h). No relationships could be established for winter, spring or summer (Fig. 2). Also, N_2 fixation rates and R_{dark} correlated positively for *Caulerpa* during spring ($F = 7.81$, $r^2 = 0.566$, $p = 0.03$; data not shown), while no other correlations for R_{dark} were found. A positive relationship was found between N_2 fixation rates and P_{net} for *Caulerpa* across all seasons, while no such relationship was found for *Lobophora*. However, the positive relationship was mainly due to 2 high values, so that no relationship was found when these 2 values were excluded.

In response to environmental parameters, positive relationships were revealed for *Lobophora*-associated N_2 fixation for temperature and irradiance, while negative relationships were found for DIN and DIP (Table 2). For *Caulerpa*-associated N_2 fixation, linear regression analysis revealed a significant positive relationship only with irradiance (Table 2). No relationships were found for P_{net} with any environmental parameters for both macroalgae (Table 2). In addition, a positive relationship was found for R_{dark} and DIP availability in *Caulerpa*, while no relationships were found for R_{dark} in *Lobophora* (Table 2).

DISCUSSION

Macroalgae-associated N_2 fixation and primary production

Previous studies reported macroalgae–diazotroph interactions for pelagic and benthic red, brown, and green macroalgae from temperate seas and tropical coral reefs as a ubiquitous and important physiological symbiosis (Carpenter 1972, Head & Carpenter 1975, Penhale & Capone 1981). In some of these studies, a single diazotroph species was isolated and tested for N_2 fixation rates (Carpenter 1972, Head & Carpenter 1975). Here, N_2 fixation rates and primary production associated with the whole consortium of 2 coral reef macroalgal holobionts are reported, i.e. the eukaryotic host and its associated diazotrophic community.

The present study found no differences in annual averaged N_2 fixation between the 2 macroalgae, i.e.

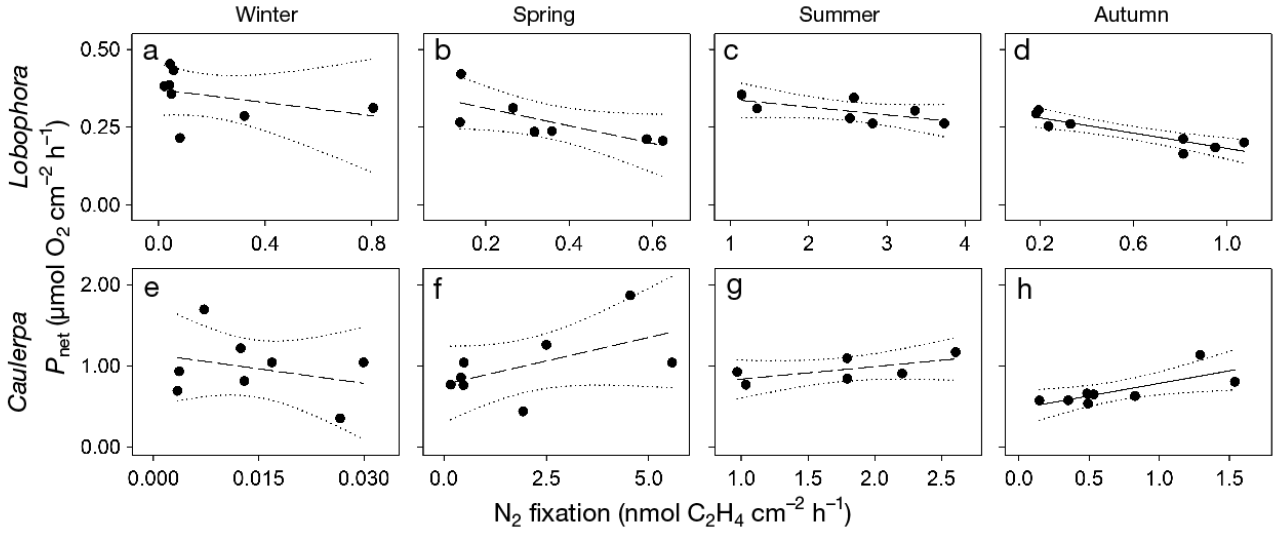

Fig. 2. Seasonal relationships between N_2 fixation and net primary productivity (P_{net}) for *Lobophora* for (a) winter, (b) spring, (c) summer, and (d) autumn, and *Caulerpa* for (e) winter, (f) spring, (g) summer, and (h) autumn (n = 6 to 8). Best-fit linear regression lines: (——) significant relationship was established; (– – –) not significant; (·····) ± 95% confidence intervals. Note the different values of all *x*-axes

Table 2. Linear regression analysis (r^2 values) for N_2 fixation (nmol C_2H_4 cm^{-2} h^{-1}), net photosynthesis (P_{net}; µmol O_2 cm^{-2} h^{-1}) and dark respiration (R_{dark}; µmol O_2 cm^{-2} h^{-1}) rates of 2 macroalgae, *Caulerpa* and *Lobophora*, and 4 different environmental water parameters, irradiance, temperature, dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorous (DIP). Bold values indicate significant positive relationships, and italicized values indicate significant negative relationships. *p < 0.05, ***p < 0.001

	Irradiance	Temperature	DIN	DIP
Caulerpa				
N_2 fixation	**0.216***	0.015	0.011	0.011
P_{net}	0.041	0.024	0.051	0.080
R_{dark}		0.059	0.121	**0.167***
Lobophora				
N_2 fixation	**0.472*****	**0.656*****	*0.517****	*0.347****
P_{net}	0.034	0.005	0.031	0.085
R_{dark}		0.013	0.031	0.045

Caulerpa and *Lobophora*, suggesting similar yearly activity of the diazotrophic community. To the best of our knowledge, this is the first study to normalize N_2 fixation activity to macroalgal surface area, whereas previous studies normalized to dry weight of the macroalgae (e.g. Carpenter 1972, Hamersley et al. 2015), making comparisons challenging. However, when comparing dry weight normalized N_2 fixation rates of brown and green algae, Capone et al. (1977) also found similar N_2 fixation rates between the green alga *Halimeda* and brown alga *Padina*, while in the same study, they found that the green alga *Microdictyon* and brown alga *Dictyota* were also similar to each other but had 5 to 10 times higher rates. Thus, rates observed here may not necessarily be representative for other green and brown algae found in the northern Red Sea.

In contrast, primary production differed between the macroalgal genera. This may be due to different preferred light regimes necessary for green and brown algae since they have evolved associations with different pigments that allow for optimal photosynthesis at different depths (Fong & Paul 2011) and thus caused by a natural physiological boundary. Furthermore, morphological differences between both genera could also explain these differences. Algae such as *Caulerpa* with a more complex or filamentous morphology have improved uptake of nutrients due to their surface area:volume ratio and can therefore exhibit higher primary production rates (Rosenberg & Ramus 1984, Gacia et al. 1996). Furthermore, *Lobophora* may favor protection from herbivores over higher primary production. High uptake of N and subsequent high primary production may come at the expense of anti-herbivory strategies as high N uptake is negatively correlated with the production of phlorotannins (Arnold et al. 1995).

Relationship between N_2 fixation and primary production

Related studies using similar methods at the same location in the Red Sea found positive correlations between N_2 fixation and parameters of primary pro-

duction (i.e. P_{net}, gross photosynthesis and/or R_{dark}) of the associated benthic organism (Bednarz et al. 2015a, Cardini et al. 2015, Rix et al. 2015). In this study, in a seasonal comparison, both N_2 fixation and P_{net} declined for both macroalgae during the transition from summer to autumn. Indeed, linear regression confirmed a relationship between the 2 processes during autumn for both macroalgae. Although the energy-demanding process of N_2 fixation is highly dependent on photosynthesis as an energy source (Mortenson 1964), the correlation in *Lobophora* was negative. This may suggest, for this algal species, a diazotrophic community dominated by non-heterocystous diazotrophs that cannot fix N_2 when oxygen is produced as a byproduct of photosynthesis (Dugdale et al. 1964, Carpenter 1972). In contrast, *Caulerpa* revealed a positive relationship between N_2 fixation and P_{net}, suggesting the presence of either heterocystous cyanobacteria, or diazotrophs with other mechanisms in place to protect the nitrogenase enzyme from oxygen inhibition. Despite the strong decline of N_2 fixation rates for *Caulerpa* from summer to autumn, the significant drop in P_{net} was not as strong as expected. This may be due to ambient DIN concentrations increasing during autumn. The DIN: DIP ratio remained below the 16:1 Redfield ratio, indicating that N remained the limiting nutrient in all seasons. This suggests that the combined concentrations of ambient DIN and bioavailable N provided by the diazotrophic community are responsible for maintaining fairly stable primary production rates in *Caulerpa* (Capone 1996, O'Neil & Capone 2008). In addition, R_{dark} correlated with N_2 fixation rates for *Caulerpa* during spring when N_2 fixation rates were high, while no correlations were found for *Lobophora*. For *Caulerpa*, R_{dark} declined significantly from spring to summer while N_2 fixation rates remained similar. In contrast, R_{dark} for *Lobophora* remained stable from spring to summer, while N_2 fixation rates increased dramatically in summer. This suggests either differences in diazotrophic activity or community structure between seasons for both macroalgae.

Seasonal patterns in N_2 fixation and primary production

Macroalgae-associated N_2 fixation rates revealed high sensitivity to seasonally changing environmental conditions, whereas the net primary productivity of the macroalgae exhibited only very minimal seasonal change. The overall N_2 fixation rate pattern of *Lobophora* revealed highest N_2 fixation rates during summer when irradiance and ambient water temperature were highest and nutrient availability (DIN and DIP) lowest. This was further substantiated by linear regression as a correlation was found for each of these parameters. Head & Carpenter (1975) also found a positive correlation between N_2 fixation rates and light intensity in the green macroalga *Codium fragile*. They also reported reduced primary production and N_2 fixation rates in shaded conditions. Here, N_2 fixation rates were positively correlated with irradiance, while no correlation was found for primary production. Thus, light intensity may have been saturating throughout the year for primary production (Franklin et al. 1996). Substantially lower rates of N_2 fixation were found during winter, spring and autumn when nutrient availability was higher. Indeed, N_2 fixation rates are likely to be affected by availability of DIN in particular (Head & Carpenter 1975, Knapp 2012). While our results report relationships on the scale of a single genus, the same patterns can be found on the community level. Overall community rates of benthic N_2 fixation appear to be strongly affected by seasonality, while primary production remains fairly similar (Cardini et al. 2016b). Moreover, this characteristic is not limited to salt water systems, as this has also been observed in oligotrophic Arctic freshwater lakes (Gettel et al. 2013). Interestingly, like in the present study, Gettel et al. (2013) also found that primary production remained fairly similar under different N_2 fixation rates. Thus, it is most likely that N_2 fixation rates in *Lobophora* used in this study were primarily regulated by nutrient availability and temperature, which can also have a positive effect on the nitrogenase enzyme (Cardini et al. 2014).

The observed pattern of N_2 fixation for *Caulerpa* was similar to *Lobophora* with the exception of the spring season. In *Caulerpa*, highest N_2 fixation rates were found in spring and in summer. Moreover, like *Lobophora*, a positive correlation between N_2 fixation and irradiance was found for *Caulerpa*. However, N_2 fixation rates during spring were not significantly different from summer, when irradiance was highest, and autumn, when irradiance was at its lowest. It is likely that the high variability during spring confounded the data, causing a lack of correlation for all other parameters. This may have been caused by physiological differences between the sampled tissues of the macroalgal genotypes, due to age of the blades (Perkins et al. 2016), or the presence/absence of heterocystous diazotrophs. Moreover, this high variability may also be explained by inhibition of nitrogenase activity due to the presence of a higher ammonium con-

centration (Sohm et al. 2011), while at the same time nitrogenase activity may be increased due to higher water temperatures and irradiance (Cardini et al. 2014). This observed pattern disparity between *Caulerpa* and *Lobophora* suggests that diazotrophic communities may differ interspecifically while the high variability in spring for *Caulerpa* even suggests large intraspecific differences (sensu Barott et al. 2011).

The seasonal pattern of N_2 fixation rates observed for *Lobophora* show a striking similarity to the one observed for coral rock investigated in parallel (Rix et al. 2015), including the apparent discrepancy during spring. Interestingly, the pattern observed for *Caulerpa* was similar to the one observed for carbonate sand investigated in parallel (Bednarz et al. 2015b). Both macroalgae were sampled from these respective substrates. Linear regression analysis, using mean N_2 fixation rates (±SE) per season from coral rock (data taken from Rix et al. 2015) and carbonate sand (data taken from Bednarz et al. 2015b) compared to N_2 fixation rates for both macroalgae measured in this study, shows a clear significant correlation (r^2 = 0.8748; $p < 0.001$) (Fig. 3). This strongly suggests that the diazotrophic community structures of both macroalgae are similar to their associated substrate (sensu Dobretsov et al. 2006).

The apparent (synergistic) links of temperature, light, and nutrient availability with N_2 fixation rates reported here highlight the complexity of eukaryote–diazotroph (macroalgal holobiont) interactions with their environment, even within a single functional group (i.e. macroalgae) or a single species/genus. Identification, relative abundance, and activity measurements of the total microbial and diazotrophic community, and also for the macroalgae-associated substrates, may shed some light on observed N_2 fixation patterns during all 4 seasons.

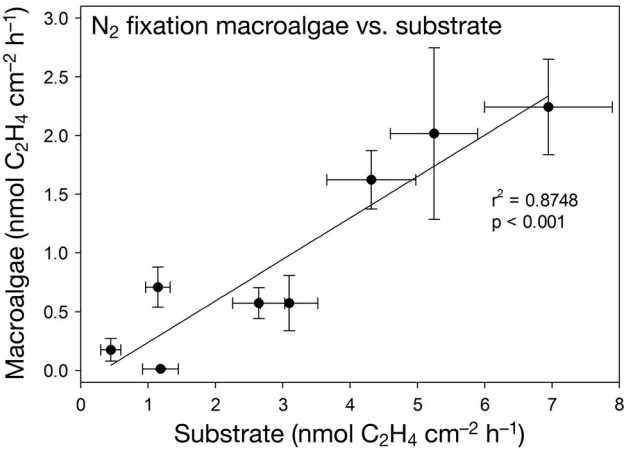

Fig. 3. Linear regression analysis of N_2 fixation rates of macroalgae (*Lobophora* and *Caulerpa*) and their associated substrate (coral rock and carbonate sand) throughout the year. Substrate data combines coral rock data from Rix et al. (2015) and carbonate sand data from Bednarz et al. (2015b). Values are given as mean ± SE

Comparison with parallel investigated organisms

The yearly macroalgae-associated N_2 fixation rates reported in the present study (9.56 ± 3.95 and 7.81 ± 4.03 µmol C_2H_4 cm^{-2} yr^{-1} for *Caulerpa* and *Lobophora*, respectively) were higher compared to hard and soft corals investigated in parallel (Table 3). N_2 fixation rates were lowest for Xeniidae at 0.18 ± 0.12 µmol C_2H_4 cm^{-2} yr^{-1} and highest for *Goniastrea* sp. (now *Coelastrea* sp.; Huang et al. 2014) at 2.14 ± 1.03 µmol C_2H_4 cm^{-2} yr^{-1} (Table 3), resulting in 9 and up to 53 times higher N_2 fixation rates in the investigated macroalgae. These differences could be attributed to abundances, community structure, and/or metabolism of diazotrophs associated with each organism (Barott et al. 2011). Overall, primary pro-

Table 3. Comparison of N_2 fixation activity and net primary production associated with different benthic coral reef organisms from the northern Red Sea. The mean value of each season was used to calculate the annual average (mean ± SE, n = 4)

Reef organism	N fixation activity (µmol C_2H_4 cm^{-2} yr^{-1})	Net primary production (mmol O_2 cm^{-2} yr^{-1})	Reference
Green alga *Caulerpa* sp.	9.56 ± 3.95	7.88 ± 1.44	This study
Brown alga *Lobophora* sp.	7.81 ± 4.03	2.63 ± 0.37	This study
Hard coral *Acropora* sp.	0.91 ± 0.73	5.03 ± 0.66	Cardini et al. (2015)
Hard coral *Pocillopora* sp.	1.07 ± 0.44	4.54 ± 0.81	Cardini et al. (2015)
Hard coral *Stylophora* sp.	1.38 ± 0.67	4.21 ± 0.93	Cardini et al. (2015)
Hard coral *Goniastrea* sp.	2.14 ± 1.03	5.38 ± 0.74	Cardini et al. (2015)
Soft coral Xeniidae	0.18 ± 0.12	3.43 ± 0.50	Bednarz et al. (2015a)
Soft coral *Sarcophyton* sp.	0.52 ± 0.24	1.86 ± 0.71	Bednarz et al. (2015a)
Sponge *Mycale* sp.	1.72 ± 1.07	–2.10 ± 0.65	Rix et al. (2015)
Turf algae	40.09 ± 19.35	4.59 ± 0.91	Rix et al. (2015)

duction rates of all benthic organisms compared by this study were relatively similar (Table 3). However, in corals, large quantities of photosynthetic products (dissolved organic carbon [DOC]) are transferred to the coral host (e.g. Muscatine 1973, Muscatine & Porter 1977). Thus, less DOC may be available for the coral-associated microbial community. Macroalgae can release considerably higher proportions of DOC compared to corals (Haas et al. 2013), which could be particularly beneficial as an energy source for (epibiotic) heterotrophic microbes. Furthermore, because of its composition, DOC released by macroalgae can cause considerably higher growth of microbes compared to DOC released by corals (Nelson et al. 2013). At the same time, epibiotic diazotrophs on macroalgae are usually exposed to good light conditions that may provide an optimal light regime for photosynthetic diazotrophs, including cyanobacteria (Barott et al. 2011). Thus, we suggest that macroalgae are a more favorable host for diazotrophs compared to corals, providing optimal conditions for both heterotrophic (algae-derived DOC as energy source) and autotrophic (light as energy source) species.

N_2 fixation rates presented in this study were lower compared to turf algae investigated in parallel, which had N_2 fixation rates of 40.09 ± 19.35 µmol C_2H_4 cm^{-2} yr^{-1}, while rates for the macroalgae were up to 5 times lower (Table 3). This could be explained by (a combination of) 2 reasons. Firstly, these differences could be attributed to turf algae assemblage structure and characteristics, such as high turnover and opportunistic growth dynamics (Littler et al. 2006, Littler & Littler 2013). Given these life-history traits, it is not unlikely that the diazotrophic part of the turf algae assemblage displays similar characteristics, i.e. fast growth and high activity to provide bioavailable N to facilitate growth of the assemblage. Secondly, even though P_{net} rates found in this study are relatively similar to those in turf algae, release of organic matter in the form of DOC is considerably higher in turf algae (compared to macroalgae) and also subject to seasonality (Haas et al. 2010). Surprisingly, Haas et al. (2010) reported lowest DOC release rates during summer, while the N_2 fixation rates reported by Rix et al. (2015) were highest during summer. Besides eukaryotic algae, turf algae assemblages may consist of high numbers of filamentous cyanobacteria (den Haan et al. 2014). However, high abundances of heterotrophs can be found in these cyanobacterial mats (Zehr et al. 1995). Thus, instead of being released in the water column, it is likely that released DOC is rapidly utilized by the microbial community (and thus not measurable), providing energy for N_2 fixation.

Implications for coral–algal phase shifts

With sea surface temperatures expected to exceed coral temperature thresholds more often in the future (Hoegh-Guldberg et al. 2007), following the potential subsequent mass mortality of corals due to bleaching, phase shifts from coral- to algae-dominated reefs are more likely to occur. Moreover, recent research has revealed that benthic algae, such as *Lobophora*, can rapidly utilize excess nutrients from terrestrial run-off and thrive under these conditions (den Haan et al. 2016), while corals, depending on the type of eutrophication, possess reduced resilience (Wiedenmann et al. 2012, Vega Thurber et al. 2014). Thus, eutrophication can result in the loss of coral cover and give rise to potential spaces for (macro)algae to grow. Also, following disturbances, (macro)algae can rapidly colonize new territory (Hughes 1994), possibly facilitated by higher N_2 fixation rates compared to e.g. hard corals. *Lobophora* in particular can occupy substrates otherwise available for coral recruits (Kuffner et al. 2006) and can cause coral mortality by shading (Box & Mumby 2007). This study and that of Rix et al. (2015) reveal that N_2 fixation rates in macroalgae, as well as turf algae, were significantly higher than those measured in corals investigated in parallel, while their primary production was similar (Table 3). The high variability of N_2 fixation rates observed in turf algae assemblages (Rix et al. 2015) makes it difficult to determine whether they are able to outcompete the macroalgae investigated in the present study. Thus, a qualified statement on competition between turf and macroalgae cannot be made.

N_2 fixation rates appeared to be affected by DIN with lower diazotrophic activity under less oligotrophic conditions. These findings indicate that warming-induced phase shifts from corals to (macro) algae could result in increased diazotrophic-driven import of N in tropical shallow coastal environments. In perspective, this may suggest that ocean warming and a subsequent increase in the input of fixed N by diazotrophs could result in phase shifts even in the absence of anthropogenic eutrophication. In addition, this input of fixed N could potentially facilitate an increase in DOC release by turf algae (Mueller et al. 2016), creating a positive feedback loop that can be detrimental to coral health status (Bourne et al. 2009, Haas et al. 2010, Rädecker et al. 2015). Although increased ammonium concentrations downregulate N_2 fixation activity during and after a phase shift, diazotrophs may act as an important trigger for changing the ecosystem.

In conclusion, while both climate change-related stressors and eutrophication can cause phase shifts, the results presented here indicate that the type of disturbance is likely to influence N_2 fixation rates differently. Whereas increased sea surface temperatures (partly) correlated with increased N_2 fixation rates, so did declining ambient DIN availability. The apparent role of DIN in the N_2 fixation rates reported here and in related literature suggest that input of N from allochthonous sources may cause N_2 fixation to become an obsolete biological mechanism for producing bioavailable N, and also for macroalgae during phase shifts. Our study further suggests that ocean warming may be accompanied by higher diazotroph activity associated with macroalgae, but only while oligotrophic conditions persist.

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