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Seasonality in dinitrogen fixation and primary productivity by coral reef framework substrates from the northern Red Sea

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ABSTRACT: N_2 fixation by coral reef benthic substrates may support primary productivity on oligotrophic coral reefs. However, little is known regarding the influence of environmental parameters on coral reef benthic N_2 fixation. This study quantified N_2 fixation and photosynthesis in 3 common reef framework substrates: turf algae, coral rock, and the abundant encrusting sponge *Mycale fistulifera* over 4 seasons in the northern Gulf of Aqaba. N_2 fixation activity was detected during day and night for all substrates, but on an annual average was significantly higher for turf algae (4.4 ± 3.9 nmol C_2H_4 cm^{-2} h^{-1}) and coral rock (3.5 ± 2.8 nmol C_2H_4 cm^{-2} h^{-1}) compared to *M. fistulifera* (0.2 ± 0.2 nmol C_2H_4 cm^{-2} h^{-1}). There was strong seasonality in N_2 fixation, with rates one order of magnitude higher in summer when temperature and irradiance were highest but inorganic nutrient concentrations lowest. During summer and fall, when nutrients were low, we found a significant positive linear relationship between gross photosynthesis (P_{gross}) and N_2 fixation in turf algae and coral rock. Further, we estimate N_2 fixation can supply up to 20 and 27% of the N demand for net photosynthesis (P_{net}) in coral rock and turf algae, respectively. By contrast there was no significant relationship between N_2 fixation and P_{gross} in *M. fistulifera*, which displayed negative P_{net} and heterotrophic metabolism (P_{gross}:respiration <1). These findings highlight the role of environmental parameters in regulating benthic substrate-associated N_2 fixation and the potential importance of fixed N for supporting primary production, particularly during nutrient-depleted conditions.

KEY WORDS: Acetylene reduction · Photosynthesis · Turf algae · Sponges · Coral rock · Gulf of Aqaba

INTRODUCTION

Coral reefs are characterized by high productivity but are typically surrounded by oligotrophic waters (Odum & Odum 1955, Hatcher 1988), where nitrogen (N) is a key limiting nutrient for growth (Delgado & Lapointe 1994, Eyre et al. 2008). Efficient internal nutrient recycling in the benthos contributes to this high productivity, but input of new N is essential to sustain net ecosystem production and growth. Though energetically costly, numerous studies demonstrate that dinitrogen (N_2) fixation represents a substantial source of new N on coral reefs (Webb et al. 1975, Larkum et al. 1988, O'Neil & Capone 1989, Charpy et al. 2007).

Biological N_2 fixation is carried out by a diverse group of heterotrophic and photoautotrophic bacteria (Zehr et al. 2003), but cyanobacteria are a key

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contributor to benthic N_2 fixation on coral reefs (Casareto et al. 2008, Charpy et al. 2012). Cyanobacterial mats have attracted much research focus due to their high N_2 fixation rates (e.g. Charpy et al. 2007, Bauer et al. 2008, Casareto et al. 2008). However, cyanobacteria are also important components of the various reef framework substrates that are ubiquitous on coral reefs; including algal turfs, endolithic algal communities associated with calcium carbonate structures, and endosymbiotic communities of sponges (Charpy et al. 2012). In coral reefs in the northern Gulf of Aqaba, these reef framework substrates are a dominant component of the benthos. Turf algae make up 72% of the benthic community on reefs in Eilat (Israel), while on the Jordanian side of the Gulf, biogenic reef framework with only sparse epilithic overgrowth (hereafter: coral rock) can account for up to 58% of the benthic cover (Bahartan et al. 2010). Sponges meanwhile dominate the cryptic reef habitat (Richter et al. 2001). High rates of N_2 fixation have been measured in both turf algae and coral rock (e.g. Larkum et al. 1988, Williams & Carpenter 1998); therefore these reef framework substrates may make important contributions to fixed N on reefs in the Gulf of Aqaba. Evidence for active N_2 fixation in sponges is scarce (Wilkinson & Fay 1979, Shashar et al. 1994a, Shieh & Lin 1994), but many species harbor microbial symbionts capable of fixing N (Taylor et al. 2007, Mohamed et al. 2008, Fiore et al. 2015) suggesting N_2 fixation in sponges may be widespread.

Due to their association with photosynthetic cyanobacteria and algae, reef framework substrates also contribute to reef photoautotrophic primary production. Turf algae are dominant primary producers on many reefs (Adey & Goertemiller 1987, Carpenter & Williams 2007) and more than one-third of sponges in the Caribbean, Great Barrier Reef (GBR) and West Indian Ocean harbor photosynthetic symbionts (Wilkinson 1987, Steindler et al. 2002, Erwin & Thacker 2007). Due to the oxygen (O_2) sensitivity of nitrogenase, the enzyme responsible for N_2 fixation, photosynthesizing diazotrophs have evolved strategies to allow photosynthesis and N_2 fixation to co-occur (Berman-Frank et al. 2003). Spatial separation in heterocystous cyanobacteria allows the fixation of N_2 during the day (Gallon 2001), while non-heterocystous cyanobacteria typically fix N_2 at night, relying on energy derived from the carbon (C) fixed during the previous daylight period (Bergman et al. 1997, Charpy et al. 2007). In marine sponges, hypoxic zones may facilitate O_2-sensitive processes such as N_2 fixation (Hoffmann et al. 2005). N_2 fixation may provide an additional source of N to support benthic primary production; however, little is known regarding the interaction between N_2 fixation and photosynthesis in benthic substrates, and few studies have quantified both processes in parallel (Williams & Carpenter 1997, Charpy et al. 2007, Lesser et al. 2007, Casareto et al. 2008).

Fringing reefs in the Gulf of Aqaba experience strong seasonal variation in key environmental parameters as a result of the annual stratification cycle in the water column. Winter and early spring are characterized by low temperature and irradiance but high inorganic nutrient concentrations as deep convective mixing of the water column transports nutrient-enriched deep water into the photic zone (Carlson et al. 2014). Increasing irradiance followed by warming sea surface temperatures throughout spring and summer lead to the development of a thermocline >100 m deep (Carlson et al. 2014) with a nutrient-depleted surface layer (Silverman et al. 2007). This results in summer conditions of high temperature and irradiance but low inorganic nutrient concentrations. Near-surface temperatures range from 21 to 28°C throughout the year while inorganic nutrient concentrations can vary by an order of magnitude (Silverman et al. 2007, Carlson et al. 2014). Such environmental parameters are known to influence planktonic N_2 fixation (Sohm et al. 2011), but their effect on N_2 fixation by benthic reef diazotrophs is largely unknown (Cardini et al. 2014).

The objectives of this study, therefore, were (1) to quantify rates of N_2 fixation and primary productivity (i.e. photosynthesis) in 3 dominant reef framework substrates (turf algae, coral rock, and an abundant encrusting sponge) over successive 4 seasons in order to evaluate the effect of seasonally variable key environmental parameters on these processes, and (2) to estimate the contribution of N_2 fixation to the N requirements for primary production in the 3 investigated substrates.

MATERIALS AND METHODS

Study site

This study was conducted in the northern Gulf of Aqaba at the Marine Science Station (MSS) Aqaba, Jordan (29° 27′ N, 34° 58′ E). Sampling was carried out on the 1 km long fringing reef in front of the MSS, which is designated as a marine reserve, and experimental work was carried out in the MSS laboratories. In order to examine the effect of seasonality, all ex-

periments were repeated over 4 seasonal periods in 2013: winter (February), spring (April), summer (September), and fall (November).

The benthic reef community was dominated by hard and soft corals (58.1 ± 13.8%), while coral rock represented the third most abundant benthic substrate type at 10 m water depth, covering on an annual average 14.2 ± 5.0% of the available substrate. The percent cover of turf algae (annual average: 4.4 ± 4.5%) was seasonally variable, reaching a maximum of 10.3 ± 4.2% in winter and decreasing to a minimum of 1.0 ± 1.0% in fall. Sponge cover was constant throughout the year, averaging 1.2 ± 0.9%. The non-cryptic sponge community was dominated by the abundant encrusting sponge *Mycale fistulifera*, which accounted for 65% of the visible sponge cover at 10 m water depth. Together the 3 investigated substrates accounted for 19.8 ± 10.3% of the total benthic coverage.

Environmental monitoring

During each season *in situ* water temperature and irradiance were continuously monitored over 4 wk at 1 min intervals at the sampling site using a data logger (Onset HOBO Pendant UA-002-64; temperature accuracy: ±0.53°C, spectral detection range: 150–1200 nm) placed in an unshaded position on the reef at 10 m water depth. Parallel irradiance measurements with a quantum sensor (Model LI-192SA; Li-Cor) allowed the conversion of lux measurements to photosynthetically active radiation (PAR, µmol photons m^{-2} s^{-1}, wavelength 400–700 nm) using a conversion factor of 1 µmol quanta m^{-2} s^{-1} = 52 lux. Irradiance data are presented as seasonal means (± SD) of daily maximum values (µmol quanta m^{-2} s^{-1}) and integrated diurnal values (mol quanta m^{-2} d^{-1}; see Table 1). Weekly seawater samples (n = 4 per season) were collected by SCUBA using acid-washed high-density polyethylene canisters (n = 4, 5 l) at 10 m water depth (~1 m above the bottom) and immediately transferred to the laboratory for further processing. Subsamples (n = 4) were taken for quantification of inorganic nutrients, particulate organic carbon (POC), particulate nitrogen (PN), and chlorophyll *a* (chl *a*). Inorganic nutrient subsamples were syringe-filtered through cellulose acetate filters (nominal pore size 0.45 µm) for determination of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and phosphate (PO_4^{3-}) concentrations using standard methods, although a modification of the cadmium column method for NO_3^- determination using a cadmium sponge was used to enable field analysis (Murphy & Riley 1962, Strickland & Parsons 1972, Holmes et al. 1999). NH_4^+ was determined fluorometrically using a Trilogy Fluorometer (Turner Designs), while all other nutrients were measured photometrically with a JASCO-V630 spectrophotometer (Jasco Analytical Instruments). The detection limits for NH_4^+, PO_4^{3-}, and NO_3^- + NO_2^- were 0.09, 0.01, and 0.02 µM, respectively. Subsamples for chl *a* determination (n = 4, 1 l) were filtered onto pre-combusted (4 h at 450°C) GF/F filters (VWR: nominal pore size 0.7 µm) and stored frozen at −80°C in the dark until further processing. Chl *a* was extracted with 90% acetone (12 h in the dark at 4°C) and analysed fluorometrically using a Trilogy fluorometer fitted with the non-acidification module (CHL NA #046, Turner Designs). Subsamples for POC (1 l) and PN (2 l) were filtered onto pre-combusted GF/F filters and dried in the oven (48 h at 40°C). Prior to analysis, POC filters were decalcified with 0.1 N HCl. POC and PN filter contents were measured on a EuroVector elemental analyzer (EURO EA 3000) with analytical precision of ≤0.1% (C) and ≤0.03% (N) using the elemental standard Acetanilide OAS (certificate 187560).

Substrate collection and maintenance

Samples of the 3 investigated reef framework substrates; turf algae, coral rock, and the encrusting sponge *M. fistulifera*, were collected from the reef at 10 m water depth by SCUBA and immediately transferred to the aquarium facility without air exposure. Turf algae were defined as a thick mat consisting of a heterogeneous assemblage of filamentous algae, crustose coralline algae (CCA), and filamentous cyanobacteria. In the Gulf of Aqaba, turf algae are composed predominately of Phaeophyta and Rhodophyta of the order Ceramiales as well as green algae of the genus C*ladophora* and cyanobacteria (Bahartan et al. 2010, Haas et al. 2010). Coral rock was considered biogenic reef framework lacking coverage by a single dominant visible epilithic group with the carbonate structure clearly visible and open for settling organisms. In the Gulf of Aqaba this hard substrate can cover large areas of the reef and is commonly referred to as 'bare rock' or 'bare substrate' (e.g. Shashar et al. 1994a, Bahartan et al. 2010), although it is associated with endolithic algae, epilithic microbial biofilms, and sparse patches of CCA, cyanobacteria and filamentous algae (Bahartan et al. 2010, Charpy et al. 2012). *M. fistulifera* is an encrusting sponge approx. 0.2 to 0.5 cm thick, typically found encrusting coral skeletons. Turf algae and *M. fistulif-*

era were collected by chiseling small pieces of dead branching corals overgrown by either turf or *M. fistulifera*, and were attached to ceramic tiles with coral glue (Reef Construct, Aqua Medic®) to minimize stress during experimental handling. Coral rock was sampled by chiseling pieces of reef framework. For each substrate, 8 replicates per season were collected with a mean height of 5 to 6 cm and mean surface area of 33.5 ± 18.9 cm^2. Specimens were maintained in an outdoor 1000 l flow-through tank supplied with seawater pumped directly from the reef at 10 m water depth at a rate of approx. 4000 l h^{-1}, ensuring key environmental parameters (e.g. temperature and inorganic nutrient concentrations) corresponded to seasonal *in situ* conditions. Irradiance (PAR) was adjusted to *in situ* levels at 10 m water depth with layers of black mesh. Parallel irradiance measurements with a quantum PAR sensor (Model LI-192SA; Li-Cor) *in situ* at 10 m and in the maintenance tank ensured irradiance corresponded to seasonal *in situ* conditions (see Table 1) with the following standard deviations: 21.8, 56.4, 26.4, 19.6 µmol photons m^{-2} s^{-1} for winter, spring, summer and fall, respectively. All incubation measurements were conducted in the same maintenance tank to ensure consistent temperature and irradiance values. *M. fistulifera* specimens were allowed to heal and acclimate for 1 wk prior to experiments and only healthy, actively pumping specimens were incubated. Turf algae and coral rock were collected 24 h before incubations were conducted.

Quantification of dinitrogen fixation

N_2 fixation rates were quantified using the acetylene (C_2H_2) reduction assay method (Capone 1993, Wilson et al. 2012). Specimens (n = 8 per substrate) were incubated in individual 1000 ml transparent glass chambers containing 800 ml of natural seawater and 200 ml headspace. Organisms were transferred into chambers without air exposure and positioned to ensure comparable irradiances in all chambers. Immediately prior to the start of the incubations, 10% of the seawater was replaced with C_2H_2-saturated seawater. Chambers were then sealed gastight with a spring-loaded glass lid and 10% of the 200 ml headspace was replaced with freshly generated C_2H_2 gas via a needle injection port in the glass lid. Sealed chambers were stirred with magnetic stirrers (600 rpm) and positioned in the flow-through tank to ensure *in situ* temperature and irradiance throughout the 24 h incubation period. Parallel measurements of irradiance inside the chambers and in the flow-through tank revealed no significant differences, ensuring that irradiances inside the chambers corresponded to the seasonal values as shown in Table 1. Incubations started and ended just prior to sunset (approx. 17:00 h) and gas samples were taken after 0, 4, 12, 16 and 24 h, except during spring when samples were taken only at 0 and 24 h. These sampling intervals were selected to capture the periods of dusk, night, dawn, and full daylight as N_2 fixation during low light conditions (dawn and dusk) is a strategy by some diazotrophs to manage the oxygen inhibition of nitrogenase (Lesser et al. 2007). At each time interval 1 ml of gas sample was collected from the headspace of each chamber with a gastight syringe and transferred into gastight 2 ml glass vials fitted with butyl septa and filled with distilled water. Vials were stored frozen upside down until analysis.

Ethylene (C_2H_4) concentrations in the gas samples were measured using a reducing compound photometer (RCP) (Peak Laboratories) with a detection limit of 100 ppb. Calibration of the RCP was routinely conducted using serial dilutions of a 200 ppm (±2%) C_2H_4 standard in air (Restek). Differences in C_2H_4 concentration between the time intervals of the incubation period were converted into C_2H_4 evolution rates according to Breitbarth et al. (2004). The C_2H_4 concentrations of the samples were corrected for the signal of unfiltered seawater controls (n = 8) and normalized to incubation time and surface area of the specimen in order to calculate C_2H_4 evolution rates (nmol C_2H_4 cm^{-2} h^{-1}). Additional controls for 0.2 µm filtered seawater (n = 6), unfiltered seawater and ceramic tile (n = 6), and unfiltered seawater with specimens but no addition of C_2H_2 (natural C_2H_4 production, n = 6), showed negligible C_2H_4 evolution. Surface areas were measured using a standard geometric technique (Advanced Geometry) as described by Naumann et al. (2009). To convert C_2H_4 evolution rates to N_2 fixation rates, a conservative theoretical ratio of 4:1 (C_2H_4:N_2) was used, which assumes that 4 mol of C_2H_4 are reduced per 1 mol of N_2. This is more conservative than the theoretical stoichiometric ratio of 3:1 as it accounts for the inhibition of the hydrogenase reaction of nitrogenase under C_2H_4-reducing conditions (Capone 1993, Mulholland et al. 2004).

Quantification of primary productivity

Primary productivity (i.e. photosynthesis) was quantified via dissolved O_2 fluxes. Substrates and seawater controls (n = 8 replicates each) were incubated in individual 1000 ml airtight transparent glass chambers

filled with natural seawater and sealed with a transparent glass lid. The sealed chambers were incubated under identical conditions as described above for N_2 fixation measurements. Incubations for respiration (R) were conducted 1 to 2 h after sunset in complete darkness for 90 to 120 min. Incubations for net photosynthesis (P_{net}) were carried out between 12:00 and 14:00 h the following day during maximum light intensity for 60 to 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). Start O_2 concentrations were subtracted from end O_2 concentrations to quantify P_{net} and R. O_2 fluxes were corrected for the mean O_2 difference found in the seawater controls and normalized to incubation time and surface area of the respective specimen. R is presented as a positive rate and gross photosynthesis (P_{gross}) rates were calculated as: $P_{gross} = P_{net} + R$.

To calculate the contribution of fixed N to the N demand for primary production, O_2 fluxes were converted into dissolved inorganic C fluxes using a photosynthetic quotient (PQ) of 1.04 and respiratory quotient (RQ) of 0.96 for turf algae and coral rock (Carpenter & Williams 2007). Since no literature values were available for marine sponges, a PQ/RQ of 1 was used for *M. fistulifera*. It was assumed that turf algae and *M. fistulifera* assimilate biomass with C:N ratios of 13.7 ± 1.3 and 6.2 ± 0.3, respectively, based on C and N elemental analyses of macroalgae and *M. fistulifera* from the study site (L. Rix unpubl. data). Since no data were available for coral rock from the Gulf of Aqaba, C:N ratios of epi- and endolithic algae associated with coral rubble from Le Reunion and Sesoko Islands were used (9.7 ± 1.5; Casareto et al. 2008). These reefs also belong to the Indo-Pacific and display comparable inorganic nutrient concentrations (Casareto et al. 2008), and were therefore deemed representative. However, variations in community assemblages may result in corresponding variations in tissue C:N ratios. Nevertheless, our intention is to provide a mainly qualitative estimate of the importance of N_2 fixation for primary production by demonstrating how much new N is made available by N_2 fixation that could potentially be used to meet the demand for net primary production and biomass generation.

Statistical analysis

The influence of 'season' and 'substrate' on all physiological parameters was estimated using fully crossed general linear models fitted in R v.3.1.1 (R Development Core Team 2014). A second model was run examining the effect of 'season' and 'substrate' and 'time of day' (day or night) on N_2 fixation. Season was used as a fixed factor encompassing the combined effects of all environmental parameters. The influence of individual environmental parameters was further examined using linear regressions. To confirm the assumptions of normally distributed and homogenous residuals, qq plots and scatter plots of residuals against fitted values were visually inspected (Quinn & Keough 2002), and data were log-transformed where necessary. Model stability was checked by examining leverage and Cook's distance as well as dffits and dfbetas, and all values were deemed acceptable. Model significance was tested using likelihood ratio tests, comparing the deviances of full models with those of the null models comprising only the intercept. The significance of individual factors was tested by removing the factor of interest and comparing the deviance to the respective full models. If factors were found to be significant, pairwise post-hoc comparisons (*t*-tests) were used to check the comparisons of interest.

RESULTS

Environmental monitoring

All environmental parameters monitored showed marked seasonal variability over the study period, with the most pronounced differences occurring between winter and summer (Table 1). Both the daily maximum irradiance and integrated diurnal irradiance were higher in spring and summer compared to winter and fall (Table 1). *In situ* temperature at 10 m water depth ranged from 22.4 to 28.0°C over the year, remaining low throughout winter and spring, then reaching a maximum in summer before decreasing again in fall (Table 1). Concentrations of NH_4^+, NO_3^- + NO_2^- and PO_4^{3-} exhibited a negative correlation with temperature (lm: all $p < 0.001$), with concentrations more than twice as high in winter and spring compared to summer and fall, reflecting the deep winter mixing and summer stratification of the water column (Silverman et al. 2007). The ratio of dissolved inorganic nitrogen to phosphate (DIN:PO_4^{3-}) ranged from 3.4 to 15.6 over the year but was consistently lower than the Redfield ratio (16:1), while POC:PN ratios always exceeded the Redfield ratio (106:16), indicating a deficiency of N compared to Redfield proportions. Chl *a* decreased by half in summer compared to all other seasons, while POC and PN were highest in spring during the seasonal plankton bloom (Table 1).

Table 1. Environmental parameters (mean ± SD) monitored at 10 m water depth over 4 seasonal periods in 2013: winter (February), spring (April), summer (September), and fall (November). Parameters include irradiance measured as both the mean daily maximum (µmol photons m^{-2} s^{-2}) and integrated diurnal mean (mol photons m^{-2} d^{-1}), temperature, ammonium (NH_4^+), nitrate + nitrite (NO_3^- + NO_2^-), total dissolved inorganic nitrogen (DIN), phosphate (PO_4^{3-}), particulate organic carbon (POC), particulate nitrogen (PN), and chl a. Temperature and irradiance were measured continuously during each seasonal period while other parameters were measured once weekly over 4 wk (n = 4)

Parameter	Winter	Spring	Summer	Fall
Daily maximum PAR	180 ± 43	252 ± 38	307 ± 25	171 ± 20
Integrated diurnal PAR	3.43 ± 0.66	5.71 ± 0.32	7.25 ± 0.47	3.51 ± 0.47
Temperature (°C)	22.5 ± 0.1	22.8 ± 0.3	27.5 ± 0.2	25.2 ± 0.2
NH_4^+ (µM)	0.32 ± 0.09	0.46 ± 0.11	0.14 ± 0.07	0.28 ± 0.07
NO_3^- + NO_2^- (µM)	0.79 ± 0.16	0.49 ± 0.19	0.09 ± 0.21	0.18 ± 0.05
DIN (µM)	1.11 ± 0.19	0.96 ± 0.08	0.23 ± 0.07	0.46 ± 0.10
PO_4^{3-} (µM)	0.11 ± 0.01	0.10 ± 0.02	0.04 ± 0.02	0.04 ± 0.02
DIN:PO_4^{3-}	10.50 ± 1.09	9.68 ± 0.43	8.10 ± 3.40	12.93 ± 2.22
POC (µM)	6.33 ± 0.70	10.25 ± 0.72	7.96 ± 1.35	8.81 ± 2.10
PN (µM)	0.85 ± 0.07	1.27 ± 0.05	0.96 ± 0.28	0.87 ± 0.37
POC:PN	7.34 ± 1.15	8.18 ± 1.29	8.34 ± 1.17	10.20 ± 1.62
Chl *a* (µg l^{-1})	0.21 ± 0.02	0.22 ± 0.04	0.10 ± 0.04	0.19 ± 0.04

Dinitrogen fixation

N_2 fixation activity varied significantly by substrate, season, and an interaction between the 2 factors (Fig. 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/m533p079_supp.pdf). On annual average, N_2 fixation was significantly higher in turf algae (4.4 ± 3.9 nmol C_2H_4 cm^{-2} h^{-1}) and coral rock (3.5 ± 2.8 nmol C_2H_4 cm^{-2} h^{-1}) compared to *Mycale fistulifera* (0.2 ± 0.2 nmol C_2H_4 cm^{-2} h^{-1}) (post hoc paired *t*-test: both p < 0.001). N_2 fixation of turf algae and coral rock were similar on annual average but significantly higher for turf algae in winter and summer, although in summer this was due to higher nighttime N_2 fixation by turf algae (post hoc paired *t*-test: all p < 0.001). N_2 fixation rates for all substrates were significantly and up to an order of magnitude higher in summer compared to all other seasons (post hoc paired *t*-test: all p < 0.001) (Fig. 1). Coral rock and *M. fistulifera* also displayed significantly lower N_2 fixation activity in winter (post hoc paired *t*-test: all p < 0.05), with the winter N_2 fixation activity in *M. fistulifera* not significantly different from seawater controls. There were no significant differences in N_2 fixation between spring and fall for any substrate (Fig. 1). Irradiance and temperature had a positive effect on N_2 fixation, while inorganic nutrient concentrations had a negative effect (Table 2). Irradiance explained more variation in N_2 fixation in turf algae and *M. fistulifera* than in coral rock, and for all substrates DIN explained more variation in N_2 fixation than PO_4^{3-} concentrations (Table 2).

N_2 fixation activity also varied significantly by time of day (Fig. 2, Table S2 in the Supplement). However, dawn and dusk values did not differ significantly from either the day or night values, and therefore we present only the day and night rates (Fig. 2). *M. fistulifera* exhibited significantly higher N_2 fixation during the day compared to the night in all 3 seasons

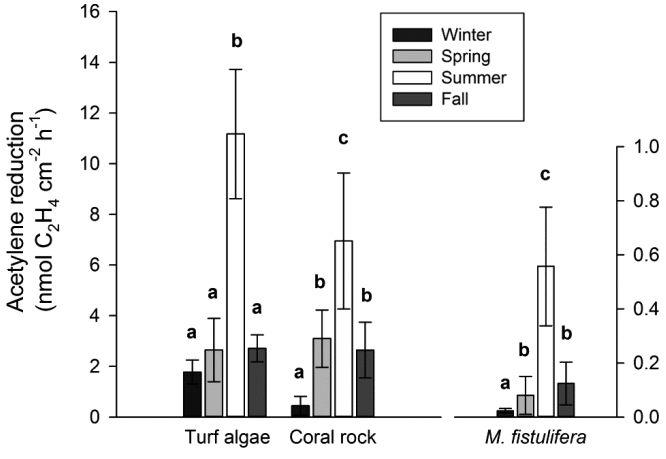

Fig. 1. Mean N_2 fixation measured as acetylene reduction rates of the 3 investigated benthic substrates over the 4 seasonal periods in 2013; winter (Feb), spring (Apr), summer (Sep), fall (Nov). Values are mean ± SD (n = 8). Different letters indicate statistical differences within each substrate. Note the different *y*-axis scale for *Mycale fistulifera*

Table 2. Linear regression analysis (presented as R^2 values) of the influence of key environmental parameters on the P_{gross} and N_2 fixation rates of the 3 investigated substrates. **Bold** values indicate a significant positive linear relationship and *italicized* values indicate a significant negative linear relationship. P_{gross} = gross photosynthesis, DIN = dissolved inorganic nitrogen, PO_4^{3-} = phosphate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not significant

	Irradiance	Temperature	DIN	PO_4^{3-}
N_2 fixation				
Turf algae	**0.542*****	**0.696*****	*0.586****	*0.399****
Coral rock	**0.415*****	**0.458*****	*0.511****	*0.396****
Mycale fistulifera	**0.503*****	**0.714*****	*0.696****	*0.584****
P_{gross}				
Turf algae	**0.505*****	**0.424*****	*0.305***	*0.163**
Coral rock	0.028^{ns}	0.122^{ns}	0.096^{ns}	0.088^{ns}
Mycale fistulifera	**0.403*****	**0.212****	*0.244***	*0.162**

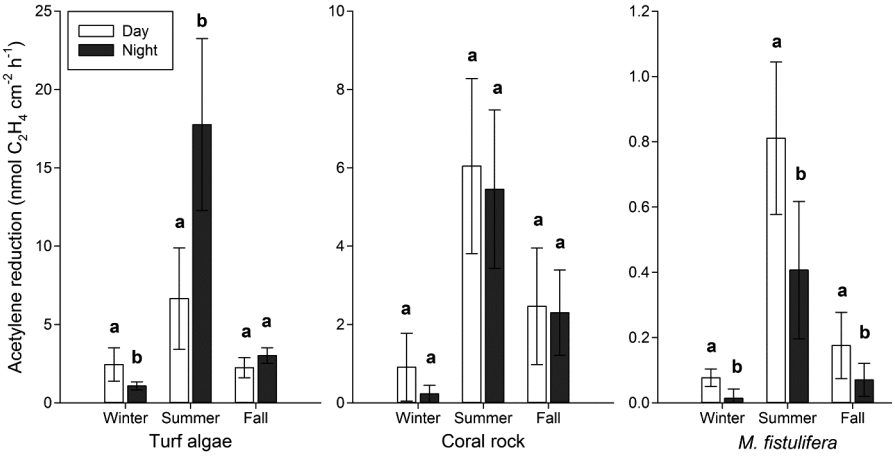

Fig. 2. Mean day and night N_2 fixation measured as acetylene reduction rates of turf algae, coral rock, and *Mycale fistulifera* over 3 seasons in 2013 (winter, summer, and fall). Values are mean ± SD (n = 8). Different letters indicate statistical differences within each substrate. Note the different *y*-axis scales

Table 3. Metabolic parameters measured in the 3 investigated substrates over 4 seasons in 2013. Rates are presented as nmol O_2 cm^{-2} h^{-1} (mean ± SD, n = 8). P_{gross} = gross photosynthesis, R = respiration, P_{net} = net photosynthesis

Season	P_{gross}	R	P_{net}	P_{gross}:R
Turf algae				
Winter	528 ± 85	95 ± 12	433 ± 77	5.6 ± 0.7
Spring	550 ± 107	78 ± 19	473 ± 95	7.2 ± 1.1
Summer	894 ± 162	119 ± 27	775 ± 155	7.8 ± 2.1
Fall	509 ± 84	95 ± 19	415 ± 68	5.4 ± 0.6
Mean	**620 ± 195**	**97 ± 24**	**524 ± 179**	**6.5 ± 1.6**
Coral rock				
Winter	472 ± 237	96 ± 55	379 ± 186	4.7 ± 0.4
Spring	354 ± 165	69 ± 38	271 ± 136	3.4 ± 1.9
Summer	553 ± 152	179 ± 95	374 ± 124	3.5 ± 1.2
Fall	446 ± 134	144 ±35	302 ± 105	3.1 ± 0.5
Mean	**438 ± 189**	**122 ± 72**	**316 ± 158**	**3.7 ± 1.9**
Mycale fistulifera				
Winter	64 ± 49	238 ± 38	−174 ± 27	0.3 ± 0.1
Spring	220 ± 42	348 ± 103	−139 ± 109	0.7 ± 0.2
Summer	307 ± 108	563 ± 55	−256 ± 80	0.5 ± 0.2
Fall	139 ± 68	564 ± 106	−393 ± 190	0.3 ± 0.2
Mean	**190 ± 121**	**431 ± 164**	**−240 ± 149**	**0.5 ± 0.3**

examined (i.e. winter, summer, and fall) (post hoc paired *t*-test: all $p < 0.01$). N_2 fixation in turf algae showed a seasonally variable response to time of day, with significantly higher daytime N_2 fixation in winter but significantly higher nighttime N_2 fixation in summer (Fig. 2). This was the only instance of significantly higher N_2 fixation at night but it was also the highest N_2 fixation rate measured over all substrates and seasons, with a rate of 17.8 ± 5.5 nmol C_2H_4 cm^{-2} h^{-1}. Coral rock displayed no significant differences in N_2 fixation between day and night (Fig. 2).

Primary productivity

There were significant effects of substrate and season as well as a significant interaction between the 2 factors for all physiological parameters measured (Table S1 in the Supplement). Over all seasons, rates of P_{gross} were significantly higher in turf algae compared to coral rock (post hoc paired *t*-test: $p < 0.001$) and significantly lower in *M. fistulifera* compared to both other substrates (post hoc paired *t*-test: both $p < 0.001$). Despite low positive P_{gross} rates, *M. fistulifera* exhibited negative P_{net} rates (Table 3). This was due to high R rates, which were significantly higher than for turf algae and coral rock (post hoc paired *t*-test: both $p < 0.001$) and resulted in low P_{gross}:R ratios (<1), indicating heterotrophic metabolism by the sponge (Wilkinson 1987). Coral rock exhibited significantly higher R and lower P_{gross}:R ratios than turf algae (post hoc paired *t*-test: both $p < 0.001$) (Table 3).

Seasonal variations in P_{gross} were less pronounced than for N_2 fixation (Table 3). P_{gross} was significantly higher in turf algae in summer (post hoc paired *t*-test: all $p < 0.001$) and significantly lower in coral rock in spring (post hoc paired *t*-test: $p < 0.01$) (Table 3). It should be noted that P_{gross} and P_{net} rates for turf algae in spring could represent the lower end of typical spring values due to unseasonably low irradiances during the turf algae photosynthesis incubations (73.8 ± 26.3 µmol photons m^{-2} s^{-1} compared to the seasonal mean of 252 ± 38 µmol photons m^{-2} s^{-1}). P_{gross} in *M. fistulifera* was significantly higher in spring compared to winter and in summer compared at all other seasons (post hoc paired *t*-test: all $p < 0.001$). Irradiance and temperature were positively correlated with P_{gross} in turf algae and *M. fistulifera* and appeared to explain more of the seasonal variation

in P_{gross} than DIN and PO_4^{3-}, which were negatively correlated (Table 2). There was little seasonal variation in P_{gross} of coral rock, with no significant effect of any of the monitored environmental parameters (Table 2).

Contribution of dinitrogen fixation to primary productivity

There was a significant positive linear relationship between P_{gross} and N_2 fixation for turf algae and coral rock only during summer and fall (Table 4). N_2 fixation explained 65 to 76% of the variation in P_{gross} in turf algae and 62 to 74% in coral rock during these seasons. In contrast, there was no significant relationship between N_2 fixation and P_{gross} in *M. fistulifera* during any of the 4 seasons (Table 4). The potential contribution of N_2 fixation to the N demand for P_{net}, which represents the new production available for growth after accounting for respiration, was on average 10.5% for turf algae and 14.5%, for coral rock, but non-calculable for *M. fistulifera*, which displayed negative P_{net} (Table 5). This contribution was seasonally variable with N_2 fixation having the potential to supply the highest amounts of N in summer (up to 19.8 and 26.8% of the N required to meet the demand for P_{net} in turf algae and coral rock, respectively), while the contributions in winter were estimated to be less than 6% for all substrates (Table 5).

Table 4. Linear regression analysis (presented as R^2 values) between the gross photosynthesis (P_{gross}) rates and N_2 fixation rates of each of the 3 substrates during each of the 4 seasonal periods. **Bold** values indicate a significant positive linear relationship. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not significant

	Turf algae	Coral rock	*Mycale fistulifera*
Winter	0.245^{ns}	0.216^{ns}	0.022^{ns}
Spring	0.064^{ns}	0.318^{ns}	0.205^{ns}
Summer	**0.653***	**0.626***	0.199^{ns}
Fall	**0.741****	**0.704****	0.374^{ns}

Table 5. Dinitrogen (N_2) fixation, net primary production, nitrogen (N) required to meet the demand for net production and the percentage of the N requirement for net production met by N_2 fixation in the 3 investigated substrates over 4 seasons. Values are mean ± SD (n = 8)

Season	N_2 fixation (μmol N cm^{-2} d^{-1})	Net production (μmol C cm^{-2} d^{-1})	N req. for net prod. (μmol N cm^{-2} d^{-1})	% N req. met
Turf algae				
Winter	0.021 ± 0.006	5.0 ± 0.9	0.36 ± 0.07	5.9 ± 1.4
Spring	0.032 ± 0.015	5.5 ± 1.1	0.40 ± 0.08	8.1 ± 3.6
Summer	0.134 ± 0.031	8.9 ± 1.8	0.65 ± 0.12	19.8 ± 3.2
Fall	0.033 ± 0.006	4.8 ± 0.8	0.35 ± 0.06	9.3 ± 0.9
Mean	**0.052 ± 0.047**	**6.0 ± 2.1**	**0.44 ± 0.15**	**10.5 ± 5.8**
Coral rock				
Winter	0.005 ± 0.004	4.4 ± 2.1	0.32 ± 0.16	2.2 ± 1.9
Spring	0.037 ± 0.014	3.1 ± 1.6	0.25 ± 0.11	12.6 ± 1.7
Summer	0.083 ± 0.032	4.3 ± 1.4	0.31 ± 0.10	26.8 ± 6.4
Fall	0.032 ± 0.013	3.5 ± 1.2	0.25 ± 0.09	12.5 ± 2.8
Mean	**0.041 ± 0.034**	**3.9 ± 1.6**	**0.28 ± 0.15**	**14.5 ± 10.0**
M. fistulifera				
Winter	0.007 ± 0.001	−2.0 ± 0.3	–	–
Spring	0.001 ± 0.001	−1.3 ± 0.8	–	–
Summer	0.007 ± 0.003	−3.0 ± 0.9	–	–
Fall	0.001 ± 0.003	−4.5 ± 2.2	–	–
Mean	**0.002 ± 0.003**	**−2.7 ± 1.7**	–	–

DISCUSSION

Dinitrogen fixation in coral reef framework substrates

N_2 fixation rates presented here are comparable to those reported for turf algae, coral rock, and sponges on coral reefs worldwide (Table 6). The relatively high variability in N_2 fixation reported for turf algae likely results from regional differences in turf community composition (Bauer et al. 2008) or responses to local environmental conditions (Williams & Carpenter 1998). While N_2 fixation rates previously reported for turf algae are typically higher than those of coral rock (Table 6), our rates for both substrates were similar, with significantly higher N_2 fixation in turf algae only during winter and summer at night. However, studies reporting higher rates of N_2 fixation in 'bare' rock compared to rock with epilithic algae, suggest endolithic N_2 fixation can exceed that of some epilithic communities (Wilkinson et al. 1984, Casareto et al. 2008). This demonstrates the importance of apparently 'bare' substrate in generating new N on coral reefs.

N_2 fixation rates in *Mycale fistulifera* were an order of magnitude lower than for turf algae and coral rock, but are in the range typically reported for other animal–microbe symbioses such as scler-

Table 6. Comparison of known N_2 fixation rates of turf algae, coral rock, and sponges reported from coral reefs worldwide. Values are presented as nmol N cm^{-2} h^{-1}. Original C_2H_4:N_2 conversion rates were used to calculate the N_2 fixation rates from acetylene reduction rates if reported in the original study. If no conversion rate was available the conservative ratio of 4:1 was used

Substrate	N fixation	Region	Reference
Turf algae	0.9–5.6	Red Sea	Present study
Turf algae	4.6 ± 0.3	Red Sea	Shashar et al. (1994a)
Turf algae	0.3–29.7[a]	Great Barrier Reef	Larkum et al. (1988)
Turf algae	8.3–36.7	Great Barrier Reef	Wilkinson & Sammarco (1983)
Turf algae	13.5 ± 5.5	Hawaiian Islands	Williams & Carpenter (1998)
Turf algae	3.7 ± 5.4	Caribbean	Williams & Carpenter (1997)
Turf algae	6.0 ± 0.9	Caribbean	den Haan et al. (2014)
Coral rock	0.2–3.5	Red Sea	Present study
Coral rock	0.3 ± 0.2	Red Sea	Shashar et al. (1994a)
Coral rock	0.2–1.9	Great Barrier Reef	Wilkinson et al. (1984)
Coral rock	0.1–6.4	Great Barrier Reef	Davey et al. (2008)
Coral rock	6.4 ± 1.8[a]	Great Barrier Reef	Larkum et al. (1988)
Coral rock	0.6[b]	French Polynesia	Charpy-Roubaud et al. (2001)
Sponge (*M. fistulifera*)	0.01–0.3	Red Sea	Present study
Sponges	0.1 ± 0.2	Red Sea	Shashar et al. (1994a)

[a]Conversion factor 3.45
[b]Conversion factor 3.3

actinian corals (Shashar et al. 1994b, Davey et al. 2008) and other cnidarians (Shashar et al. 1994a, Bednarz et al. 2015a). There are very few reports of active N_2 fixation in marine sponges, but the rates for *M. fistulifera* are consistent with those reported for other Red Sea sponges in the only other study presenting sponge N_2 fixation rates normalized to organism surface area (Shashar et al. 1994a; Table 6). It should be noted that both studies used the acetylene reduction method, which has reportedly proven problematic for some sponges (Wilkinson 1999). However, all sponges were actively pumping post-incubation and the high pumping rate would ensure rapid flushing of the tissue, excluding the likelihood of acetylene toxicity or insufficient acetylene and ethylene transport. Acetylene may disrupt other N cycling processes, such as nitrification, that occur in some sponges, however the absence of nitrate production by *M. fistulifera* suggests it does not host this process (L. Rix unpubl. data). We cannot exclude metabolism of ethylene by microbial symbionts; however, this would affect all 3 substrates and not only the sponge. It may rather be that this apparent difficulty in measuring N_2 fixation in sponges is due to in part to low N_2 fixation activity, as observed here (particularly in winter) and in other studies (Wilkinson & Fay 1979, Shashar et al. 1994a, Shieh & Lin 1994, Wilkinson 1999). Despite the low rates, we observed consistent and measureable N_2 fixation with low variability and clear seasonal trends. Further, the low $\delta^{15}N$ tissue values of *M. fistulifera* (<1‰, L. Rix unpubl. data) are consistent with biological ^{15}N fixation (Yamamuro et al. 1995, Montoya et al. 2002). The lower N_2 fixation activity in *M. fistulifera* is likely due to low diazotroph abundances or activity in the sponge-associated microbial community compared to turf algae and coral rock, which can be composed largely of N_2 fixing cyanobacteria (Charpy et al. 2012). Given that the DIN release rates reported for sponges exceed reported rates of N_2 fixation by orders of magnitude (Diaz & Ward 1997, Southwell 2007), N_2 fixation may not be of high functional importance for the nutrition of the sponge host. As efficient filter feeders sponges may meet their N demand primarily via heterotrophic feeding (Pile et al. 2003).

Diel dinitrogen fixation pattern

While many studies have found substantially higher daytime N_2 fixation activity in coral rock (Wilkinson et al. 1984, Charpy-Roubaud et al. 2001, Holmes & Johnstone 2010) and turf algae (Williams & Carpenter 1997, den Haan et al. 2014), our results show consistent and substantial nighttime N_2 fixation by both substrates. Turf algae exhibited significantly higher daytime N_2 fixation in winter but significantly (3 times) higher nighttime N_2 fixation in summer. This could indicate a shift in the turf community towards more non-heterocystous cyanobacteria or heterotrophic diazotrophs in summer, as typically only heterocystous cyanobacteria can fix N_2 in the presence of O_2 generated by photosynthesis (Bergman et al. 1997). While cyanobacteria have long been considered the primary diazotrophs responsible for benthic marine N_2 fixation, the role of heterotrophic bacteria is increasingly being recognized (Zehr et al. 1995, Bauer et al. 2008). Identification of the diazotroph community would provide further insight into the patterns of N_2 fixation observed here. Similar day and night N_2 fixation rates by coral rock suggest a diazotroph community equally adapted to light and dark conditions. Only *M. fistulifera* consistently exhibited significantly (2 times) higher N_2 fixation activity in the day compared to night, suggesting the

role of either phototrophic diazotrophs or heterotrophic diazotrophs energetically dependent on photosynthetic products. Sponges host diverse communities of microbial symbionts and nitrogen fixation *nifH* genes affiliated with a range of cyanobacteria and heterotrophic bacteria have been detected in tropical sponges (Mohamed et al. 2008, Zhang et al. 2014, Fiore et al. 2015). Evidence for active cyanobacterial *nifH* expression dominating during the day and higher proteobacterial *nifH* expression at night in the congeneric *Mycale laxissima* suggests multiple diazotrophs can contribute to sponge-associated N_2 fixation and provides a potential explanation for diel patterns of sponge N_2 fixation activity (Mohamed et al. 2008, Zhang et al. 2014). The presence of cyanobacteria could also explain our findings of P_{gross} in *M. fistulifera* and future studies should investigate the symbionts responsible for photosynthesis and N_2 fixation in the sponge. The lack of peaks in N_2 fixation during low light levels as observed for the coral *Montastrea cavernosa* (Lesser et al. 2007) and the co-occurrence of N_2 fixation during the day with photosynthesis indicate that the diazotroph communities of the 3 investigated substrates are equipped with other strategies to overcome O_2 inhibition of nitrogenase.

Seasonality in dinitrogen fixation and primary productivity

This is the first study examining seasonal N_2 fixation in a diverse group of reef framework substrates, and our findings highlight the importance of environmental parameters in regulating benthic N_2 fixation activity. Temperature and irradiance positively affected N_2 fixation, while inorganic nutrients had a negative influence, resulting in the highest rates in summer when irradiance and temperature were highest but inorganic nutrients lowest. This seasonal pattern showed remarkable consistency across the 3 substrates despite their differing trophic strategies, and is consistent with reports of higher summer benthic N_2 fixation on the GBR (Larkum et al. 1988) as well as higher summer N_2 fixation in soft corals (Bednarz et al. 2015a) and pelagic communities in the Red Sea (Rahav et al. 2015). High temperatures can directly stimulate the enzymatic activity of nitrogenase and are associated with increased growth and N_2 fixation in free-living cyanobacteria (Breitbarth et al. 2007). Conversely, lower temperatures can increase respiratory costs associated with N_2 fixation in unicellular cyanobacteria (Brauer et al. 2013). However, temperature alone likely cannot explain the observed seasonality, as there were no significant differences in N_2 fixation between spring and fall, despite a 2.5°C temperature difference. By enhancing photosynthesis, irradiance may stimulate the energetically costly process of N_2 fixation through the provision of larger quantities of energy-rich photosynthates (Bebout et al. 1993), if the responsible diazotrophs are protected from the corresponding increase in O_2 production. Although this largely appears to be the case for the substrates investigated here, given only turf algae and *M. fistulifera* exhibited significantly higher summer photosynthesis but all 3 substrates displayed an increase in N_2 fixation, irradiance was unlikely the primary driving factor. The effect of decreased DIN concentrations during summer likely played a key role and can be explained by the higher energetic costs of N_2 fixation compared to DIN assimilation (Gallon 2001), making it a seasonal strategy when external nutrients are scarce. This is supported by observations of increased nitrogenase activity in N-starved cultured filamentous cyanobacteria (Ramos et al. 1985) and inhibition of N_2 fixation in coral skeletons and reef sediments under elevated NH_4^+ concentrations (Koop et al. 2001, Holmes & Johnstone 2010). These findings suggest diazotrophs are capable of altering their N_2 fixation activity to adjust to the availability of external N sources. While elevated N_2 fixation also increases iron (Fe) demand compared to NH_4^+ assimilation (Kustka et al. 2003), Fe limitation is unlikely to be a limiting factor in the Gulf of Aqaba due to high dust inputs (Ying et al. 2007, Foster et al. 2009). Alternatively, seasonal variability in N_2 fixation activity may be influenced by seasonal changes in the diazotroph communities associated with the 3 substrates. Overall the combination of key environmental parameters in summer (i.e. high irradiance, high temperature, and low inorganic nutrients), appear to interact to cause substantially higher N_2 fixation rates. Importantly, this results in the highest N_2 fixation when the inorganic N supply is lowest.

Contribution of dinitrogen fixation to primary productivity

Interestingly, high photosynthesis rates were sustained in summer when temperature and irradiance were highest, despite low inorganic nutrient availability. This suggests that either primary production was not nutrient (DIN) limited or that additional nutrient sources contributed to supporting photosynthesis. Given the significant positive linear relation-

ship between P_{gross} and N_2 fixation for both turf algae and coral rock only during summer and fall when inorganic nutrient concentrations were low, this suggests fixed N may play a role in supporting primary production — at least when other sources of nutrients are scarce. For turf algae, we estimated that while fixed N could only supply 6% of the N demand for P_{net} in winter, this increased to 20% in summer, suggesting N_2 fixation has the potential to be an important N supply for photosynthesis. Williams & Carpenter (1997) found N_2 fixation contributed <2% to the N demand for P_{net} in turf algae in the Caribbean, with the estimated contribution by NH_4^+ assimilation an order of magnitude higher. While comparable to our winter estimates, this is substantially lower than our summer values. However, the N_2 fixation rates measured by Williams & Carpenter (1997) were lower than those measured during our study in summer, and NH_4^+ concentrations may have differed. While DIN assimilation would also represent an important process at our study site, increased summer N_2 fixation may compensate for the decrease in DIN concentrations, sustaining high summer P_{net} rates. For coral rock, we estimate that N_2 fixation could supply 2% of the N demand for P_{net} in winter and 27% in summer. This is remarkably similar to the 2 to 28% estimated for coral rock from Sesoko Island (Pacific Ocean) and Le Reunion (Indian Ocean) (Casareto et al. 2008). However, it should be noted that these calculations are highly dependent on the C:N of the substrates, which may vary spatially and temporally. Further, photosynthetic rates change over the day but here were measured only during periods of maximum irradiance. However, since these measurements represent the maximum P_{net} rates (preliminary photosynthesis-irradiance curves showed no photoinhibition at these irradiances), our estimates of the potential contribution of N_2 fixation to primary production can be considered conservative as they may underestimate the importance of N_2 fixation at lower P_{net} rates occurring over the day. Future studies should investigate the utilization of fixed N by the turf algae and coral rock consortia to confirm a relationship between N_2 fixation and primary production. In contrast to turf algae and coral rock, we found no significant relationship between N_2 fixation and P_{gross} in *M. fistulifera*. Sponges release large quantities of DIN (e.g. Southwell et al. 2008), which may provide another source of N for their photosynthetic symbionts and could explain the lack of correlation between the 2 processes found here. While photosynthesis supplies a significant portion of the energy demand of some sponge species (Wilkinson 1987, Erwin & Thacker 2007), given *M. fistulifera* exhibited negative P_{net} and overall heterotrophic metabolism (P_{gross}:R < 1), photosynthesis is unlikely to be important to its overall nutrition. Nevertheless, these findings highlight the potential for N_2 fixation to contribute to sustaining high rates of P_{net} in coral rock and turf algae during the period of water column stratification in the Gulf of Aqaba.

Ecological implications

In addition to directly supporting their own growth, N fixed by turf algae and coral rock may substantially contribute to new N on coral reefs via N release from cyanobacterial cells (Mulholland et al. 2004), mechanical disturbances such as grazing (Williams & Carpenter 1997), or by the recycling of diazotroph biomass. Using the estimated 3D surface area of each substrate per m^2 of reef, we calculate that N_2 fixation by the 3 substrates introduces to the reef 47 µmol N_2 m^{-2} d^{-1} of fixed N in winter and up to 185 µmol N m^{-2} d^{-1} in summer. These values are comparable to the benthic reef community N_2 fixation estimated for One Tree Island (GBR) of 78–156 µmol N_2 m^{-2} d^{-1} (Larkum et al. 1988), but lower than those calculated for Eilat (Red Sea) of 576–960 µmol N_2 m^{-2} d^{-1} (Shashar et al. 1994a). However, it is likely that N_2 fixation by other benthic substrates not accounted for here, such as reef sands (Charpy-Roubaud et al. 2001, Casareto et al. 2008, Bednarz et al. 2015b), cyanobacterial mats (Charpy et al. 2007), and hard corals (Lesser et al. 2007), also substantially contribute to reef N generation. Due to the low abundance of *M. fistulifera* on the studied reef and the comparatively low rates of N_2 fixation associated with the microbial community of *M. fistulifera*, it contributed <1% of the total new N fixed by the 3 investigated substrates. Unless the abundant cryptic sponge community fixes N at higher rates, sponges likely do not contribute substantially to reef N generation, at least via N_2 fixation. They do produce large quantities of inorganic nutrients through remineralization of particulate organic matter and association with nitrifying bacteria (Richter et al. 2001, Southwell et al. 2008). While turf algae are increasingly a dominant component on many coral reefs (e.g. Bahartan et al. 2010), their low and seasonally variable abundance at the present study site resulted in a substantial contribution to new reef N generation only in winter (72%). Low abundances in other seasons resulted in a contribution of 10 to 31% of the total N fixed by the 3 investigated substrates during the rest

of the year. Given their high potential for N_2 fixation, turf algae are likely an important source of new N on turf-dominated reefs (e.g. den Haan et al. 2014). High N_2 fixation and high benthic coverage (11 to 20%) characterize coral rock as the major year-round contributor of newly fixed N among the 3 investigated substrates, accounting for 28% in winter but 69 to 90% of the N fixed during the other seasons. Overall, 2 to 4 times more N was fixed in summer compared to the other seasons. This fixed N appears to be of greater ecological importance during the low-nutrient summer season in the Gulf of Aqaba, particularly for supporting primary production. This highlights the potential significance of N_2 fixation by coral rock and turf algae in coral reef ecosystems with more constant oligotrophic conditions.

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