Vol. 527: 47–57, 2015
doi: 10.3354/meps11224

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published May 7

# Dinitrogen fixation and primary productivity by carbonate and silicate reef sand communities of the Northern Red Sea

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ABSTRACT: Permeable sediments are highly bioactive compartments in coral reefs. The associated dense microbial communities sustain fast degradation of organic matter, thereby playing a key role in nutrient recycling within the reef. Besides nutrient recycling, new nutrients (i.e. nitrogen) are acquired by dinitrogen ($N_2$) fixing microbial communities, but knowledge about the influence of sand mineralogy and key environmental factors on this process is scarce. Therefore, this study quantified seasonal $N_2$ fixation (via acetylene reduction) along with gross photosynthesis (via $O_2$ fluxes) by adjacent carbonate and silicate sands in a Northern Red Sea coral reef. Findings revealed significantly higher $N_2$ fixation in carbonate than in silicate sands (2.88 and 1.52 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$, respectively) and a more pronounced seasonal response in the former, likely caused by its higher permeability, grain size and microbial abundance. Ambient light and organic matter availability were the main controlling environmental factors for sand-associated $N_2$ fixation. Carbonate and silicate sands showed similar gross photosynthesis rates (270 and 233 nmol $O_2$ $cm^{-2}$ $h^{-1}$) that positively (carbonate sands) or negatively (silicate sands) correlated with $N_2$ fixation, likely due to different diazotrophic communities. Seasonal appearance of microbial mats on carbonate sands increased $N_2$ fixation and gross photosynthesis by up to one order of magnitude. On an annual average, carbonate and silicate sands obtain ~8% and microbial mat communities obtain ~13% of their photo-metabolic N demand via $N_2$ fixation.

KEY WORDS: Carbonate sand · Silicate sand · Gulf of Aqaba · Microphytobenthos · Photosynthesis · Seasonality · Oxygen fluxes · Acetylene reduction



## INTRODUCTION

Coral reefs are characterized by high benthic community biomass and primary production despite being surrounded by oligotrophic waters (e.g. Odum & Odum 1955, Gattuso et al. 1998). Up to 90% of total carbon (C) fixation on coral reefs is derived from benthic photosynthetic primary production, where the highest production is often associated with corals (zooxanthellae), turf algae or macroalgae (Kinsey 1985, Gattuso et al. 1998). In comparison, the sand-associated microphytobenthos displays lower primary productivity rates per unit surface area, but given the often large areal extent of unconsolidated sandy sediments in reefs, net microphytobenthic primary productivity may be on the same order of magnitude and equally important as coral or macroalgal production (Kinsey 1985, Clavier & Garrigue 1999, Werner et al. 2006, Garren & Azam 2012).

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Besides primary productivity, reef sands represent an important biocatalytical filter system for organic matter (Wild et al. 2004a,b, Werner et al. 2006). The relatively large grain size of reef sands ensures high permeability ($>10^{-12}$ $m^2$) for water exchange and provides settling space for microphytobenthic communities, which both represent key factors for efficient organic matter degradation and concomitant nutrient recycling (Rasheed et al. 2003a, Wild et al. 2004a,b, Werner et al. 2006). Reef sands generally contain $10^3$ times more bacteria and up to 80 times higher nutrient concentrations than the surrounding seawater (Rasheed et al. 2002), highlighting the importance of this reef compartment for nutrient recycling in oligotrophic reef environments (Garren & Azam 2012). As oligotrophic reefs receive low amounts of allochthonous nutrient input, they strongly rely on the efficient recycling and new generation of nutrients (Howarth 1988). In particular, nitrogen (N) is mostly the limiting nutrient for primary productivity in coral reefs (Eyre et al. 2008).

Besides recycling of essential nutrients, measurements of dinitrogen ($N_2$) fixation indicate that reef sands also play an important role for the generation of new bioavailable N (Shashar et al. 1994, Charpy-Roubaud et al. 2001). Capone et al. (1992) found that $N_2$ fixation in the top layers (0 to 2 cm) of reef sediments accounted for more than 50% of the total sedimentary ammonium production. Biological $N_2$ fixation is a physiological process unique to diazotrophic prokaryotes and, despite being energy-costly, can represent an alternative nutrient supply if growing under N-limited ambient conditions typical for coral reef environments (Charpy-Roubaud et al. 2001, Scanlon & Post 2008). In coral reefs several benthic substrates (e.g. sand, coral rubble, cyanobacterial mats and living corals) are actively fixing $N_2$ (Cardini et al. 2014). Since reef sands can cover large areas on a reef, previous studies have highlighted the magnitude of sedimentary $N_2$ fixation and its importance for the N requirement of the total reef benthos (Shashar et al. 1994, Charpy-Roubaud et al. 2001, Casareto et al. 2008). Shashar et al. (1994) calculated for a lagoon in the Northern Red Sea that reef sands contribute ~70% to the total $N_2$ fixation within the reef, while Charpy-Roubaud et al. (2001) estimated that sedimentary $N_2$ fixation covers ~24% of the annual N requirements for the total benthic primary productivity in the Tikehau Lagoon (French Polynesia).

The dominant sand type in reef environments is biogenic carbonate sand, while in some regions terrigenous silicate sands co-occur. At the Northern Red Sea, the rare occurrence of flood events through otherwise desiccated river mouths lead to the deposition of silicate sands in many fringing reefs of the area. These 2 sand types are exposed to identical, seasonally variable environmental conditions but exhibit different physico-chemical characteristics in grain size, surface structure and area, permeability and transparency to light (see Table 1). Together these factors define 2 different habitats, which in turn select sand-specific microbial communities (Schöttner et al. 2011), subsequently affecting sedimentary primary productivity and $N_2$ fixation rates. Previous studies have demonstrated the importance of microphytobenthic photosynthesis and $N_2$ fixation for total benthic primary productivity and biogeochemical nutrient cycles within the reef ecosystem (Charpy-Roubaud et al. 2001, Werner et al. 2008). Nonetheless, to our best knowledge, no study has investigated both processes with particularly focus on the effect of sand mineralogy and environmental key parameters (e.g. temperature, light intensity, nutrient concentrations).

Therefore, the main objectives of the present study were (1) to quantify $N_2$ fixation and microphytobenthic photosynthesis of 3 different reef sand communities (bare carbonate sands, silicate sands and microbial mats on carbonate sands) in a seasonal resolution in order to investigate the effects of sand type along with seasonally changing environmental key parameters, and (2) to calculate the respective contribution of fixed N to the N requirements for microphytobenthic primary productivity.

## MATERIALS AND METHODS

### Study site

This study was conducted at the Marine Science Station (MSS) Aqaba in the Northern Gulf of Aqaba, Jordan (29° 27`N, 34° 58`E). The MSS is situated ~10 km south of Aqaba City with access to a Red Sea fringing coral reef inside a marine reserve. Strong regional seasonality is reflected by substantial variability of environmental key parameters throughout the year due to the annual water column stratification cycle in the Gulf of Aqaba (Silverman et al. 2007, Carlson et al. 2014). The hard coral dominated (38.6 ± 2.6%) fringing reef site reveals an average bare carbonate sand cover of 18.5 ± 2.8% with highest coverage at 5 m water depth (50.7 ± 6.3%) followed by the reef flat (19.0 ± 3.9%) and 10 m water depth (16.2 ± 1.4%). At 1 and 20 m depth, the bare carbon-

ate sand coverage is <4.0%. Overall for the site, <1% of the total bare carbonate sand area is covered by microbial mat communities throughout the year, with the highest abundance (~3%) at 5 m water depth and a seasonal development ranging from <1% in winter and summer to 5% in fall and 7% in spring. The fringing reef is interrupted by a ~100 m long area completely covered by silicate sand from the shore down to at least 40 m. This area is almost free of hard coral structures but covered 10 to 20% by seagrass beds. In order to study the effect of seasonality on $N_2$ fixation and primary productivity by microbial communities of the different reef sands, all experiments described below were conducted once in each of the following months representing a respective season: February (winter), April (spring), September (summer) and November (autumn) during the year 2013. Thermal stratification in the Gulf of Aqaba develops from May to November with a maximum during August/September, while deep-water mixing occurs from January to April reaching the maximum mixing depth in March/April (Manasrah et al. 2006).

### Monitoring of environmental parameters

*In situ* water temperature (°C) and light intensity (lux) were continuously recorded at the sampling locations (10 m depth) using data loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: ±0.53°C, spectral detection range: 150 to 1200 nm). The presented light data are seasonal means of maximum irradiance measured during 11:00 and 13:00 h (see Table 2), and lux readings were converted to photosynthetically active radiation (PAR; µmol quanta $m^{-2}$ $s^{-1}$; 400 to 700 nm wavelengths) using the following approximation: 1 µmol quanta $m^{-2}$ $s^{-1}$ = 52.0 lux. This conversion factor was obtained by inter-calibrating the lux readings with data obtained from a parallel deployed PAR sensor (LI-COR LI-192SA underwater quantum sensor) during a simultaneous minute-by-minute measurement over 5 h. Both readings correlated well ($r^2$ = 0.83) and the obtained conversion factor of 52.0 was similar to 51.2 reported by Valiela (1984). Weekly seawater samples were collected at 10 m water depth (~1 m above the sand) using high-density polyethylene canisters (5 l, n = 4) and transported back to the laboratory within 30 min. There, subsamples for inorganic nutrients, chlorophyll *a* (chl *a*), particulate organic carbon (POC) and particulate nitrogen (PN) were collected. Inorganic nutrient subsamples (50 ml) were filtered through cellulose acetate membrane filters (nominal pore size: 0.45 µm) for determination of dissolved inorganic nitrogen (DIN: ammonium, nitrate and nitrite) and dissolved inorganic phosphate (DIP) following standard methods (Murphy & Riley 1962, Strickland & Parsons 1972, Holmes et al. 1999). Ammonium was determined fluorometrically using a Trilogy Fluorometer (Turner Designs), while all other nutrients were measured photometrically with a JASCO-V630 spectrophotometer (Jasco Analytical Instruments). Detection limits for ammonium, DIP and nitrogen oxides (nitrate and nitrite) were 0.09, 0.01 and 0.02 µM, respectively. Chl *a* subsamples (1 l) were filtered onto pre-combusted GF/F filters (nominal pore size: 0.7 µm) and stored frozen at −80°C in the dark until analysis. Chl *a* was extracted with 90% acetone (12 h in the dark at 4°C) and measured using a Trilogy Fluorometer fitted with a non-acidification module (CHL NA #046, Turner Designs). Additional subsamples for POC (1 l) and PN (2 l) were filtered onto pre-combusted GF/F filters, dried in the oven (40°C, 48 h) and stored dry pending analysis. Prior to analysis dried filters were wrapped in silver foil and POC filters were acidified with 0.1 N HCl to remove any inorganic carbon. POC and PN filter contents were measured on a EuroVector elemental analyser (EURO EA 3000) with analytical precision of ≤0.1% C and ≤0.03% N.

### Substrate sampling

Two neighbouring back reef sites at 10 m water depth in front of the MSS covered by either carbonate or silicate sand were chosen for substrate sampling using SCUBA. The lateral distance between the 2 sites was ~150 to 200 m, and both sites were in close vicinity (5 m distance) to the adjacent coral reef framework. Both sand types revealed distinct mineralogical, physical and biological characteristics as repeatedly measured by several previous studies (Table 1). Once during each season, carbonate sand (n = 8) and silicate sand (n = 8) samples were taken using custom-made PVC sediment corer (inner diameter: 4.3 cm). Additional carbonate sand samples (n = 8) showing dark-brown microbial mats (~1 to 2 mm thick) on top were collected within 100 m distance from the bare carbonate sand sampling site. Cores were immediately transported back to the MSS where the top 1 cm surface layer of each core was individually transferred into a petri-dish of equal diameter (planar surface: 14.52 $cm^2$) before being placed into individual incubation glass chambers (500 ml chamber for carbonate and silicate sands,

Table 1. Sediment properties of carbonate and silicate sand in the Gulf of Aqaba previously measured at the study site. OC: organic carbon, DIN: dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate

| Parameter | Carbonate sand | Silicate sand | Reference |
|---|---|---|---|
| $CaCO_3$ content (%) | 75–87 | 4–19 | Rasheed et al. (2003b), Schöttner et al. (2011) |
| Grain size (µm) | 553–559 | 229–326 | Rasheed et al. (2003b), Schöttner et al. (2011) |
| Sorting coefficient | 1.3 | 0.9 | Rasheed et al. (2003b) |
| Permeability ($m^{-2} \times 10^{-12}$) | 116–143 | 19–27 | Rasheed et al. (2003b), Wild et al. (2005) |
| Porosity (%) | 47 | 33 | Rasheed et al. (2003b) |
| OC content (%) | 0.36 | 0.24 | Rasheed et al. (2003b) |
| OC decomposition (mg $m^{-2}$ $d^{-1}$) | 3.0 | 2.0 | Rasheed et al. (2003a) |
| DIN content (µmol $l^{-1}$) | 17–20 | 6–7 | Rasheed et al. (2003b) |
| DIP content (µmol $l^{-1}$) | 1.4–1.9 | 0.5–0.6 | Rasheed et al. (2003b) |
| Ammonium efflux (mmol $m^{-2}$ $d^{-1}$) | 3.41 ± 0.32 | 2.15 ± 0.26 | Rasheed et al. (2003a) |
| DIP efflux (mmol $m^{-2}$ $d^{-1}$) | 0.03 ± 0.002 | 0.02 ± 0.001 | Rasheed et al. (2003a) |
| Chl *a* (µg $g^{-1}$) | 0.72 ± 0.16 | 0.63 ± 0.12 | Rasheed et al. (2003b) |
| Bacterial cell number ($cm^{-3}$) | $3.1 \pm 0.9 \times 10^9$ | $1.5 \pm 0.5 \times 10^9$ | Schöttner et al. (2011) |

1000 ml chamber for microbial mats). During all handling, special care was taken to keep the sediment stratification and minimize the exposure time to air (<30 s). All chambers were kept in an outdoor 800 l flow-through aquarium during subsequent measurements of sedimentary $O_2$ fluxes and $N_2$ fixation over the next 2 d.

### Quantification of $O_2$ fluxes

All following incubations took place in the outdoor 800 l flow-through aquarium supplied with seawater pumped directly from the reef at the 10 m sampling depth (exchange rate: 4000 l $h^{-1}$) to ensure *in situ* water temperature and nutrient concentrations. Light intensity was monitored with lux and PAR data loggers (see above) and adjusted with black netting to those measured *in situ* at 10 m water depth. $O_2$ fluxes of the sand samples as a proxy for primary productivity were quantified in 2 individual incubations. The first incubation was carried out on the sample collection day 1 to 2 h after sunset to measure dark respiration ($R$), while the second incubation was started the following day at 12:00 h for net photosynthesis ($P_{net}$) determination. Each sand substrate (n = 8) was incubated individually and additional chambers (500 ml, n = 8) only filled with seawater served as controls to measure planktonic background metabolism. Chambers were sealed and incubated under constant stirring (600 rpm) for 2 to 6 h (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific™). $O_2$ concentrations were measured at the beginning and end of each incubation period using a salinity- and temperature-corrected $O_2$ optode sensor (MultiLine® IDS 3430, WTW). End concentrations never exceeded 8.3 mg $O_2$ $l^{-1}$ during $P_{net}$ nor did they fall <5.4 mg $O_2$ $l^{-1}$ during $R$ incubations. To calculate $O_2$ fluxes, $O_2$ start concentrations were subtracted from end concentrations, and the results were normalized by incubation time. Finally, $O_2$ fluxes were corrected for the seawater control signal related to the chamber volume and normalized to the sand surface area (nmol $O_2$ $cm^{-2}$ $h^{-1}$). Gross photosynthesis ($P_{gross}$) rates were calculated according to $P_{gross} = P_{net} - R$. In order to calculate the N requirement for $P_{gross}$, the daily $O_2$ production was calculated assuming a daily 12 h photoperiod and values were converted into C fluxes using a community photosynthetic (PQ) and respiratory quotient (RQ) of 1.0 (1 mol $O_2$ = 1 mol C) according to Taddei et al. (2008) who experimentally determined similar PQ and RQ values for coral reef sands.

### Quantification of $N_2$ fixation

$N_2$ fixation rates were quantified 3 to 4 h after the $P_{net}$ incubation ended by applying a modified acetylene ($C_2H_2$) reduction technique (Capone 1993, Wilson et al. 2012). $C_2H_2$ gas was freshly generated from calcium carbide and bubbled through fresh seawater in order to produce $C_2H_2$-enriched seawater. Incubations were conducted in 500 ml glass chambers containing 400 ml natural seawater of which 10% were replaced with $C_2H_2$-enriched seawater. Chambers were immediately sealed gas-tight with a spring-

loaded glass lid equipped with a rubber injection port on top for gas sampling, and 10% of the air headspace was replaced by freshly generated $C_2H_2$ gas. In addition, 4 different sets of controls were tested for the reduction of $C_2H_2$ to ethylene ($C_2H_4$) production: (1) unfiltered seawater control (without sand samples, n = 8); (2) 0.2 µm-filtered seawater control (without sand samples, n = 6); (3) petri-dish in unfiltered seawater (without sand sample, n = 6); (4) sand sample in unfiltered seawater without $C_2H_2$ addition (natural $C_2H_4$ production, n = 6). Over the entire incubation period (24 h), all chambers were magnetically stirred as described above, and gas samples were taken at 0, 4, 12, 16 and 24 h. At each of these time intervals, 1 ml of gas sample was collected with a gastight syringe from each chamber, transferred into gastight 2 ml vials previously filled with distilled water, and stored frozen upside down until analysis. $C_2H_4$ concentrations of gas samples were measured in the field laboratory using a reducing compound photometer (RCP; Peak Laboratories) with a detection limit of 100 ppb. Calibration of the RCP was conducted using serial dilutions of a 200 ± 4 ppm $C_2H_4$ standard in air (Restek). The $C_2H_4$ evolution in each incubation chamber was calculated according to Breitbarth et al. (2004). Values were finally corrected for the unfiltered seawater control signal related to the chamber volume and normalized to incubation time and sand planar surface area. All rates are reported as means ± SE and in $C_2H_4$ production rates (nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$) to allow good comparison to previous studies using the $C_2H_2$ reduction assay. $C_2H_4$ rates were only converted to $N_2$ fixation rates in order to calculate the percentage contribution by $N_2$ fixation to the N requirements for microphytobenthic primary production. Since no parallel $^{15}N$ calibration was applied, a theoretical ratio of 3 mol $C_2H_2$ reduced to 1 mol $N_2$ fixed was used, which has been previously found for white coral reef sands dominated by diatoms and dinoflagellates (Charpy-Roubaud et al. 2001).

### Statistical analysis

All statistical analyses were carried out using Primer-E version 6 software (Clarke & Gorley 2006) with the PERMANOVA+ add-on (Anderson 2001). Analyses were based on Bray Curtis similarities of the physiological parameters (square root transformed). Two-factor PERMANOVAs were performed to test for differences of the parameters $N_2$ fixation, $P_{gross}$ and $R$ rates between substrate type and season. 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 if significant differences occurred. Finally, correlations between $N_2$ fixation rates and the environmental water parameters as well as between $N_2$ fixation and sedimentary $O_2$ fluxes ($P_{gross}$ and $R$) were determined via linear regression.

## RESULTS

### Environmental key parameters

All monitored environmental key parameters exhibited strong seasonal patterns (Table 2) with the most distinct differences between the stratified (summer and fall) and the deep-water mixed (winter and spring) season. Highest irradiance (PAR) was measured in spring and summer compared to winter and fall (Table 2). Summer also revealed the highest water temperature before it decreased during fall until it reached annual minimum values during winter and spring. Inorganic nutrients (DIN and DIP) were negatively correlated to water temperature with at least twice as high concentrations during winter and spring compared to summer and fall, thereby clearly reflecting the seasonal change between stratification and deep-water mixing of the water column. The calculated DIN:DIP ratio ranged from 5.31 to 11.25 throughout the year but was consistently lower

Table 2. Summary of key environmental water parameters monitored at 10 m water depth during 4 seasons. DIN: dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate, POC: particulate organic carbon, PN: particulate nitrogen, POM (POC+PN): particulate organic matter. Values are means (n = 4) (±SE)

| Environmental variable | Winter | Spring | Summer | Autumn |
|---|---|---|---|---|
| Irradiance (PAR) | 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) |

than the Redfield ratio (16:1), indicating N limited conditions in the water column, particularly during summer. N limitation is further suggested by the ratio of POC:PN in the water column that always exceeded the Redfield ratio (106:16). POC and PN revealed highest concentrations during spring together with highest chl *a* concentrations in the water, thereby indicating the seasonal plankton bloom and the increased production of biomass during this period of the year.

### $O_2$ fluxes by reef sand communities

$P_{gross}$ rates averaged 270 ± 25 and 233 ± 17 nmol $O_2$ $cm^{-2}$ $h^{-1}$ for carbonate sand and silicate sand, respectively, across all seasons. Both bare sands exhibited similar $P_{gross}$ rates during each season except during spring when carbonate sand exhibited significantly higher rates compared to silicate sand. The seasonal pattern was similar with significantly increased $P_{gross}$ rates during spring and summer for both sands (Fig. 1, Table 3). Microbial mats showed no seasonal variation of $P_{gross}$ rates but the annual average of 809 ± 43 nmol $O_2$ $cm^{-2}$ $h^{-1}$ was 3 times higher compared to carbonate and silicate sand. *R* was on annual average almost twice as low in carbonate (–70 ± 3 nmol $O_2$ $cm^{-2}$ $h^{-1}$) compared to silicate sand (–126 ± 12 nmol $O_2$ $cm^{-2}$ $h^{-1}$). While carbonate sand showed significantly higher *R* rates during spring and summer, *R* in silicate sand peaked during winter and summer. *R* rates of microbial mats were significantly the highest during summer and averaged –135 ± 7 nmol $O_2$ $cm^{-2}$ $h^{-1}$ over all seasons, thus being in the range of *R* measured for silicate sands.

Table 3. Results of 2-factorial PERMANOVAs for $N_2$ fixation, gross photosynthesis ($P_{gross}$) and dark respiration (*R*) rates for the substrate types (carbonate sand, silicate sand and microbial mat) during the 4 investigated seasons (winter, spring, summer and autumn) in 2013. Substrate and season were fixed effects. PERMANOVA was based on Bray-Curtis similarity after square root transformation. Type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations). Significant p-values are **in bold**

| Effect | df | SS | MS | Pseudo-*F* | p-value |
|---|---|---|---|---|---|
| **$N_2$ fixation (nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$)** | | | | | |
| Substrate (Su) | 2 | 31607 | 15804 | 140.95 | **<0.001** |
| Season (Se) | 3 | 5384 | 1795 | 16.01 | **<0.001** |
| Su × Se | 6 | 11707 | 1951 | 17.40 | **<0.001** |
| Residuals | 76 | 8521 | 112 | | |
| Total | 87 | 57219 | | | |
| **$P_{gross}$ (nmol $O_2$ $cm^{-2}$ $h^{-1}$)** | | | | | |
| Substrate (Su) | 2 | 15933 | 7967 | 150.12 | **<0.001** |
| Season (Se) | 3 | 3990 | 1330 | 25.06 | **<0.001** |
| Su × Se | 6 | 2264 | 377 | 7.11 | **<0.001** |
| Residuals | 76 | 4033 | 53 | | |
| Total | 87 | 26221 | | | |
| ***R* (nmol $O_2$ $cm^{-2}$ $h^{-1}$)** | | | | | |
| Substrate (Su) | 2 | 4204 | 2102 | 44.89 | **<0.001** |
| Season (Se) | 3 | 358 | 119 | 2.55 | 0.052 |
| Su × Se | 6 | 2385 | 398 | 8.49 | **<0.001** |
| Residuals | 76 | 3559 | 47 | | |
| Total | 87 | 10507 | | | |

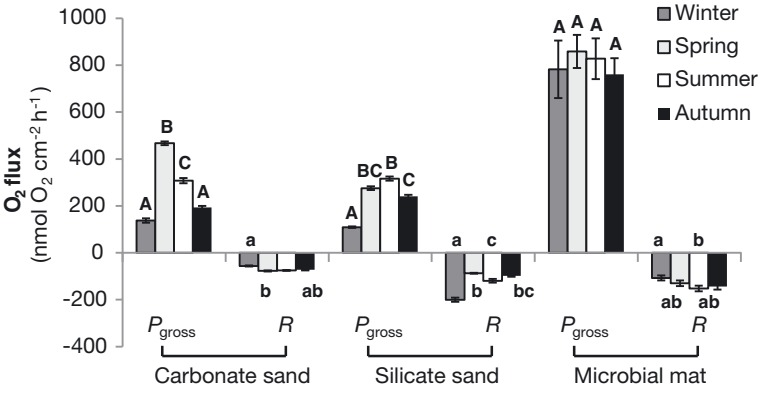


Fig. 1. Gross photosynthesis ($P_{gross}$) and dark respiration (*R*) rates measured as $O_2$ fluxes in the substrates carbonate sand, silicate sand and microbial mat during all seasons. Values: mean (n = 8) ± SE. Different letters = significant differences for $P_{gross}$ (A–C) and *R* (a–c) rates between the 4 seasons for each substrate type, respectively, based on pair-wise PERMANOVA

### $N_2$ fixation by reef sand communities

On annual average, $N_2$ fixation by carbonate sand communities (2.88 ± 0.41 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$) was significantly higher when compared to silicate sand (1.52 ± 0.15 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$). The 2 sands revealed a specific seasonal variability in $N_2$ fixation rates (Fig. 2, Table 3). Carbonate sand was significantly more active during spring and summer thereby following the seasonal pattern of $P_{gross}$. This is supported by a significant positive linear relationship with $N_2$ fixation explaining 69% of the variation in $P_{gross}$

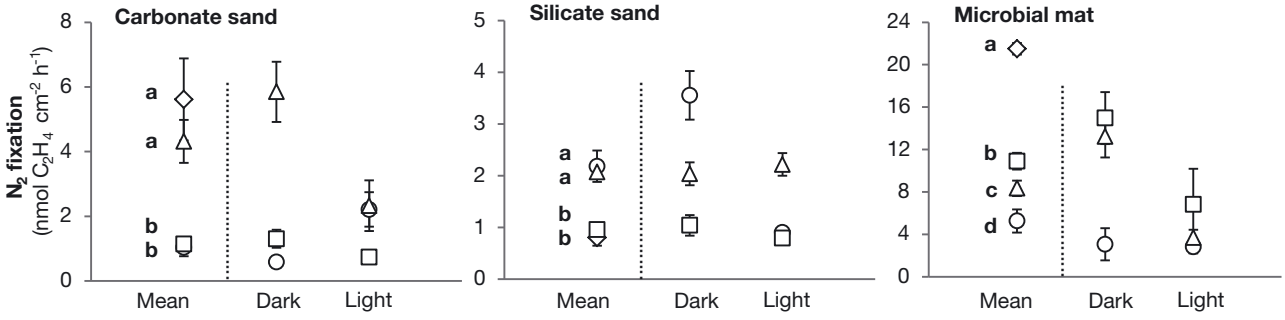


Fig. 2. Mean $N_2$ fixation ($C_2H_4$ production) rates of the different substrates (carbonate sand, silicate sand, microbial mat) measured during winter (○), spring (◇), summer (△) and autumn (□) over a 24 h incubation period. $N_2$ fixation rates for the dark and light periods are separately presented, except for spring. Values: mean (n = 8) ± SE. Different letters (a–d) = significant differences between the 4 seasons for each substrate type, respectively, based on pair-wise PERMANOVA

(Table 4). In contrast, silicate sand revealed significantly the highest $N_2$ fixation activity during winter and summer similar to seasonal maxima of *R* rates. Correlation revealed a significant positive linear relationship between the 2 processes with 38% of the variation in *R* being explained by $N_2$ fixation (Table 4). Overall, seasonal $N_2$ fixation variability was more pronounced in carbonate (1.14 to 5.25 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$) compared to silicate sand (0.81 to 2.42 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$). Correlations to the key environmental parameters for $N_2$ fixation of carbonate sand revealed a significant positive linear relationship to light intensity and POM content in the water, while $N_2$ fixation of silicate sand was negatively correlated to POM content but not to light intensity (Table 4). Additionally, $N_2$ fixation of both sands showed a significant negative relationship to the DIN:DIP ratio in the water column.

Table 4. Linear regression analysis between $N_2$ fixation rates of the 3 sand substrates (carbonate sand, silicate sand, microbial mat) and both the key environmental water parameters (DIN: dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate, POM: particulate organic matter) and the $O_2$ fluxes ($P_{gross}$: gross photosynthesis, *R*: dark respiration) of the sand substrates. Data presented as $R^2$ values at significant levels of $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$. Significant positive relationships **in bold;** significant negative relationships in *italics*

| Parameter | Carbonate sand | Silicate sand | Microbial mat |
|---|---|---|---|
| **Environmental factor** | | | |
| Irradiance | **0.491*** ** | 0.023 | 0.057 |
| Temperature | 0.045 | 0.048 | 0.052 |
| DIN | 0.017 | 0.009 | 0.043 |
| DIP | 0.003 | 0.002 | 0.033 |
| DIN:DIP | *0.189** | *0.259*** | 0.048 |
| POM | **0.212*** | *0.467**** | 0.006 |
| **Sedimentary $O_2$ fluxes** | | | |
| $P_{gross}$ | **0.690***** | *0.153** | **0.568***** |
| *R* | **0.215*** | **0.375***** | **0.610***** |

Compared to the 2 bare reef sands, $N_2$ fixation activity associated with microbial mats was always almost one order of magnitude higher (seasonal average: 11.95 ± 1.16 nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$). $N_2$ fixation in microbial mats was significantly different between each season, with the highest rates in spring, followed by fall, summer and winter (Table 3). However, no significant relationship was found between $N_2$ fixation activity and the key environmental water parameters (Table 4). Correlation analysis between $N_2$ fixation and $O_2$ fluxes in microbial mats revealed significant positive relationships to both $P_{gross}$ and *R* (Table 4).

Besides the seasonal variability of $N_2$ fixation averaged over 24 h, all 3 substrates revealed specific dark and light $N_2$ fixation rates with either similar dark and light $N_2$ fixation or relatively higher dark $N_2$ fixation on a 24 h basis (Fig. 2). Higher dark $N_2$ fixation was measured for carbonate sand during summer, for silicate sand during winter and for microbial mat communities during summer and autumn.

## DISCUSSION

### Primary productivity and $N_2$ fixation by reef sand communities

This is the first study comparatively describing primary productivity and $N_2$ fixation activity of carbonate and silicate reef sand communities. We investigated the top sediment layer where highest diazotrophic activity occurs (Werner et al. 2008). The top sediment layer of both sands can be characterized as net-autotrophic and largely independent

from allochthonous C input, as $P_{gross}$ rates largely exceeded $R$ rates. $N_2$ fixation rates for carbonate and silicate sands presented here agree well with values previously measured at different reef locations (Table 5). Shashar et al. (1994) measured higher, yet variable, $N_2$ fixation rates in reef sediments from a close site in the Gulf of Aqaba (Eilat). These differences may be explained by the use of mixed grain sizes ranging from gravel (5 mm) to fine (0.1 mm) and a higher proportion of large grain sized sands, while the present study measured $N_2$ fixation exclusively in fine grained sands (0.2 to 0.6 mm; Table 1).

The present study measured significantly higher $N_2$ fixation rates in carbonate sand than in silicate sand, and this may be explained by sediment type-specific characteristics. The coarser carbonate sand was less well sorted but had a much higher permeability and porosity than the silicate sand (Rasheed et al. 2003a, Wild et al. 2005). High permeability generates advective driven fluid fluxes between the sediment and the overlying water. This enhances solute exchange and the flux of suspended organic matter (Rasheed et al. 2003a), while a highly porous grain structure increases the specific surface area and thus the available substrate for microbial community growth. These characteristics support microbial abundance in carbonate sands that largely exceeds cell numbers in silicate sands (Wild et al. 2004a, 2006, Schöttner et al. 2011). Furthermore, significantly higher organic matter degradation and C turnover rates in carbonate sand occur (Rasheed et al. 2003a, Wild et al. 2005), which increase organic substrate availability (Table 1; Rasheed et al. 2003b). This has previously been described as a main factor controlling $N_2$ fixation activity in shallow carbonate sediments (O'Neil & Capone 1989). Since $N_2$ fixation represents an energetically costly process (due to breakage of the $N_2$ triple-bond), diazotrophs have a high need for energy-rich organic substrates, and thus may benefit from the higher organic C content in carbonate compared to silicate sands (Table 1). Furthermore, Schöttner et al. (2011) investigated microbial communities of carbonate and silicate sand in the same area and identified sand type as a main factor structuring sediment-associated microbial assemblages. Similarly, diazotrophic assemblages likely differ between the 2 sands. Overall, the present findings highlight the influential role of sediment-specific characteristics (e.g. grain size, permeability, diazotrophic composition) in controlling sediment-associated $N_2$ fixation activities.

Unconsolidated reef sands also provide open space for the development of microbial mats which are often dominated by cyanobacteria communities and represent important contributors to benthic primary productivity and N supply in coral reefs (Charpy et al. 2010, 2012, Cardini et al. 2014). The presented values for $N_2$ fixation compare well with values pre-

Table 5. Acetylene reduction (AR; nmol $C_2H_4$ $cm^{-2}$ $h^{-1}$) and inferred $N_2$ fixation rates (NF; mmol N $m^{-2}$ $d^{-1}$) of the different reef sand communities investigated in the present study in comparison with values reported from other coral reef areas worldwide. GBR: Great Barrier Reef, Australia. AR:NF is the respective $C_2H_2$:$N_2$ conversion ratio used to calculate NF from AR

| AR | AR:NF | NF | Location | Method | Reference |
|---|---|---|---|---|---|
| **Carbonate sands** | | | | | |
| 2.88 ± 0.41[a] | 3 | 0.46 ± 0.07 | Red Sea | $C_2H_2$ | Present study |
| 0.04–2.32 | 4 | 0.01–0.28 | Caribbean | $C_2H_2$ | O'Neil & Capone (1989) |
| 0.75–1.95 | 3 | 0.12–0.31 | GBR | $C_2H_2$ | Capone et al. (1992) |
| 19.52 ± 17.50[b] | 4 | 2.34 ± 2.10 | Red Sea | $C_2H_2$ | Shashar et al. (1994) |
| 0.18–1.02 | 1.8–4.8[c] | 0.03–0.28 | French Polynesia | $C_2H_2$; $^{15}N_2$ | Charpy-Roubaud et al. (2001) |
| – | – | 0.10–0.16 | Ishigaki Island | $^{15}N_2$ | Miyajima et al. (2001) |
| 0.32 | 1.6[c] | 0.34 | French Polynesia | $C_2H_2$; $^{15}N_2$ | Charpy-Roubaud & Larkum (2005) |
| 9.76 ± 3.21[a] | 4 | 1.17 ± 0.39 | New Caledonia | $C_2H_2$ | Charpy et al. (2007) |
| 0.03–0.12 | 3 | 0.004–0.019 | GBR | $C_2H_2$ | Werner et al. (2008) |
| **Silicate sands** | | | | | |
| 1.52 ± 0.15[a] | 3 | 0.24 ± 0.02 | Red Sea | $C_2H_2$ | Present study |
| **Microbial mats** | | | | | |
| 11.95 ± 1.16[a] | 3 | 1.91 ± 0.19 | Red Sea | $C_2H_2$ | Present study |
| 2.7–47.8 | 4 | 0.3–5.7 | California | $C_2H_2$ | Paerl et al. (1993) |
| 0.96 | 1.6[c] | 0.57 | French Polynesia | $C_2H_2$; $^{15}N_2$ | Charpy-Roubaud & Larkum (2005) |
| 0.59–2.97 | 4 | 0.07–0.36 | Indian Ocean | $C_2H_2$ | Charpy et al. (2012) |

[a]Mean ± SE; [b]mean ± SD; [c]conversion factor was empirically determined

viously reported for benthic microbial mats in other coral reef ecosystems (Table 5). Compared to bare carbonate sand, $N_2$ fixation and $P_{gross}$ rates of microbial mats were ~4.5 and ~3 times higher, respectively, thus indicating a higher de novo input of N relative to photosynthetically fixed C. This increased N availability may enable rapid accumulation of biomass and the formation of dense mats in an extremely oligotrophic environment. This is supported by the present study displaying highest microbial mat development and abundance during spring, the season also showing the highest year-round $N_2$ fixation activity by the mats. Nevertheless, all microbial mats in the study site were of small size and overall covered <1% of the bare carbonate sand area on the reef. Considering such low coverage by microbial mats compared to bare carbonate sand (18% of total reef area), the contribution of bare reef sand areas to total benthic $N_2$ fixation is likely much higher despite the lower fixation rates per unit of surface area.

### Seasonal variability of primary productivity and $N_2$ fixation

This study investigated the response of sediment-associated primary productivity and $N_2$ fixation to seasonal changing environmental conditions. Carbonate and silicate sands were exposed to similar changing environmental conditions, thus differences in the biological variables reflect a sand type specific response. Overall, seasonal variability was more pronounced in carbonate than in silicate sands. This is most likely due to sand-specific differences in permeability, specific surface area, microbial community and mineralogy leading to tighter benthic–pelagic coupling between the water column and sediment pore-water in carbonate sand. Therefore, seasonal variation in water column nutrient availability will more directly affect the nutrient inventory in the upper sediment layer (0 to 2 cm) of carbonate than silicate sand (Rasheed et al. 2003b). Schöttner et al. (2011) investigated the effects of season, sediment depth and location on microbial community structure in reef sediments in the Gulf of Aqaba and found that season was the most significant structuring factor in carbonate sands, while sediment depth was more influential in silicate sands. Seasonality and sediment depth may also determine the diazotrophic community structure, thus explaining the stronger seasonal variation in $N_2$ fixation activity observed for carbonate compared to silicate sand in the present study.

$N_2$ fixation in carbonate sand was primarily stimulated during spring and summer by seasonally increased ambient light and POM availability. This agrees with previous studies describing light as a main factor influencing sedimentary $N_2$ fixation (Charpy-Roubaud et al. 2001, Charpy et al. 2007, Werner et al. 2008) and suggests the dominance of phototrophic diazotrophs. The increased $N_2$ fixation rates in carbonate sand are mainly due to elevated diazotrophic activity during night, indicating a shift towards a more non-heterocystous bacterial community. Non-heterocystous diazotrophs separate the $O_2$-sensitive $N_2$ fixing nitrogenase enzyme complex temporally from $O_2$ producing photosynthesis, whereas heterocystous diazotrophs can fix $N_2$ also during daylight in specialized $O_2$-free cells (heterocyst). Nighttime $N_2$ fixation activity also depends on a photosynthetic energy supply and correlates positively to the intensity of the previous daylight period (Charpy et al. 2007). Furthermore, $N_2$ fixation activity heterotrophically profits from available organic C sources. Thus, the 2-fold higher POM supply via sedimentation during spring and summer (Wild et al. 2009) likely provides additional energy for sediment-associated $N_2$ fixation. Despite seasonal changes in POM availability, carbonate sand communities revealed little seasonal variation in $R$ rates, while primary productivity responded similarly as $N_2$ fixation to seasonality. This is in line with previous studies (Rasheed et al. 2002, 2003b, Wild et al. 2009) and suggests that the microphytobenthos is largely independent from allochthonous C input and likely sustains its primary productivity via $N_2$ fixation.

$N_2$ fixation in silicate sand was negatively correlated to $P_{gross}$, positively to $R$ and was not influenced by ambient light availability; thus it strongly indicates the dominance of heterotrophic diazotrophs. Although activity of heterotrophic diazotrophs completely relies on external organic C sources, $N_2$ fixation in silicate sand was negatively related to POM concentrations in the water column. This implies a minor organic C supply and trophic link between the sediment and the overlaying water and is further supported by a slower transport of organic substrates through the rather diffusion-limited silicate sands compared to the highly advection-driven carbonate sands (Rasheed et al. 2003b). Despite a sand-specific seasonal response, $N_2$ fixation of both sands negatively correlate to the DIN:DIP water column ratio. The low DIN:DIP ratio over the year indicates N limited conditions and suggest $N_2$ fixation as an advantageous strategy for sedimentary primary productivity.

### Contribution of $N_2$ fixation to primary productivity

The significant linear correlation between $N_2$ fixation and $P_{gross}$ suggests a tight coupling between the 2 processes. Averaged over all seasons, daily $P_{gross}$ in carbonate sand, silicate sand and microbial mats was calculated to require 4.89, 4.21 and 14.66 mmol N $m^{-2}$ $d^{-1}$, respectively, assuming the Redfield ratio (106:16) for primary productivity applicable to microphytobenthic communities of reef sands (Delesalle et al. 1998, Charpy-Roubaud et al. 2001, Werner et al. 2008). Thus, on annual average, $N_2$ fixation rates measured here would supply 8.4, 8.1 and 13.3% of the total N needed for microphytobenthic primary productivity in carbonate sand, silicate sand and microbial mats, respectively. These estimates are similar to a New Caledonian reef lagoon, where $N_2$ fixation in reef sands and microbial mat communities contributed between 5 and 21% of the N required for primary productivity (Charpy et al. 2007, 2010). Also at Sesoko, Japan, similar contributions of 5.7% for sandy bottoms and 10.0 to 26.5% for microbial mats were calculated (Casareto et al. 2008). However, these estimates likely underestimate the contribution of $N_2$ fixation, as a substantial quantity of N is recycled (autochthonous N-input) within the reef sediments (Crossland et al. 1991, Charpy-Roubaud et al. 2001) thereby largely reducing the photometabolic demand for 'new' N (allochthonous N-input) but increasing the relative N input via $N_2$ fixation.

DIN fluxes from the sediment to the overlaying waters were shown to importantly fuel primary productivity of the whole reef benthos (Charpy-Roubaud et al. 1996, 2001, Rasheed et al. 2002). At the study site, carbonate sand shows a 2.8 higher DIN content and 1.6 higher ammonium efflux to the overlaying water compared to silicate sand, which may be explained by its generally higher $N_2$ fixation and organic matter degradation rates (Table 1, Rasheed et al. 2003a,b). Overall, the present findings highlight the significant role of $N_2$ fixation as an important N source for sedimentary primary productivity. By releasing large quantities of fixed N to the overlaying water, reef sediments, particularly carbonate sands, may significantly support primary productivity of other benthic organisms and of the entire coral reef ecosystem.

*Acknowledgements.* We thank L. Rix, F. Al-Horani and S. Basuoni for field-work assistance and logistical support, and M. Birkicht, D. Peterke and S. Wilson for their help with sample analysis. This work was funded by German Research Foundation (DFG) grant Wi 2677/6-1 with support of the German Leibniz Association (WGL) and a PhD stipend from Evangelisches Studienwerk Villigst e.V. to V.N.B.

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*Editorial responsibility: Ronald Kiene, Mobile, Alabama, USA*

*Submitted: October 17, 2014; Accepted: January 29, 2015*
*Proofs received from author(s): April 2, 2015*