Contrasting seasonal responses in dinitrogen fixation between shallow and deep-water colonies of the model coral *Stylophora pistillata* in the northern Red Sea

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Abstract

Tropical corals are often associated with dinitrogen (N₂)-fixing bacteria (diazotrophs), and seasonal changes in key environmental parameters, such as dissolved inorganic nitrogen (DIN) availability and seawater temperature, are known to affect N₂ fixation in coral-microbial holobionts. Despite, then, such potential for seasonal and depth-related changes in N₂ fixation in reef corals, such variation has not yet been investigated. Therefore, this study quantified seasonal (winter vs. summer) N₂ fixation rates associated with the reef-building coral *Stylophora pistillata* collected from depths of 5, 10 and 20 m in the northern Gulf of Aqaba (Red Sea). Findings revealed that corals from all depths exhibited the highest N₂ fixation rates during the oligotrophic summer season, when up to 11% of their photo-metabolic nitrogen demand (CPND) could be met by N₂ fixation. While N₂ fixation remained seasonally stable for deep corals (20 m), it significantly decreased for the shallow corals (5 and 10 m) during the DIN-enriched winter season, accounting for less than 2% of the corals’ CPND. This contrasting seasonal response in N₂ fixation across corals of different depths could be driven by 1) release rates of coral-derived organic matter, 2) the community composition of the associated diazotrophs, and/or 3) nutrient acquisition by the *Symbiodinium* community.

Introduction

Scleractinian corals are effectively composed of an assemblage of diverse organisms (often referred to as the coral ‘holobiont’) including the cnidarian host, endosymbiotic dinoflagellates (of the genus *Symbiodinium*), bacteria, archaea and fungi [1]. *Symbiodinium* provides the heterotrophic coral host with carbon (C)-rich photosynthates that are essential for host survival...
in oligotrophic reef environments, where access to heterotrophic C sources is often limited [2]. However, net coral growth also requires a sufficient supply of nitrogen (N), another limiting nutrient in tropical reef waters [3]. In order to cope with the limited N availability, corals can acquire dissolved inorganic nitrogen (DIN) from surrounding seawater (even at very low concentrations) and have evolved efficient internal N cycling between the coral host and its photosynthetic symbionts [4–6]. In addition, corals are associated with dinitrogen (N₂)-fixing bacteria (diazotrophs) that are able to convert dissolved elemental N₂ into ammonium via nitrogenase activity [7,8]. Thus, diazotrophs may compensate for the limited DIN availability in oligotrophic reef waters by providing an additional source of N that can be assimilated and metabolized by the coral host [7,9–12].

Corals harbor both autotrophic and heterotrophic diazotrophs whose N₂ fixation activity largely depends on the prevailing environmental conditions [13]. Elevated temperature stimulates N₂ fixation in corals [14], likely by increasing the enzymatic activity of nitrogenase [15]. Conversely, high environmental DIN concentrations can decrease N₂ fixation, as the process is metabolically costlier for diazotrophs than DIN assimilation [16]. Another key factor regulating N₂ fixation, particularly in autotrophic diazotrophs, is ambient light availability [17]. Although autotrophic diazotrophs require light for photosynthesis, high levels of photosynthesis-derived oxygen (O₂) can inhibit the O₂-sensitive nitrogenase enzymes [18]. On relatively high-latitude coral reefs—such as those of the northern Red Sea (e.g. 29˚N for reefs in Jordan’s Gulf of Aqaba)—temperature, DIN concentrations and light availability differ significantly across seasons [19,20]. Previous studies on coral-associated diazotrophs in the northern Red Sea report highest N₂ fixation rates during summer, when light levels and temperature are highest and DIN concentrations are lowest [12,21]. Cardini et al. (2015) concluded that diazotrophically-derived N sustains the high primary productivity of corals during nutrient-depleted summer conditions (<0.1 μM DIN) by contributing up to 11% of the corals’ photosynthetic nitrogen demand (CPND), as opposed to only 2% during winter.

However, key abiotic parameters do not only change over temporal scales, but also over spatial scales such as along bathymetric and depth gradients [3]. On tropical coral reefs, light penetration decreases most rapidly to ~20 m, while temperature and inorganic nutrient concentrations stay constant within this depth range [22,23]. Corals undergo several adaptations in response to reduced light attenuation, such as morphological changes to optimize light harvesting [24], a shifting reliance from autotrophic to heterotrophic food sources [25,26] and changes in the associated Symbiodinium community [26,27]. The coral-associated diazotrophic community also undergoes changes along bathymetric gradients, with differences already apparent between 5 and 15 m depth [28,29]. Since diazotroph assemblages can differ across depths, the overall N₂ fixation activity associated with these coral holobionts is also hypothesized to vary across depths. In addition, diazotroph assemblages located at different depths could also be hypothesized to demonstrate variable N₂ fixation rates across seasons, especially given the aforementioned temporal changes in DIN levels. A recent study using the ¹⁵N₂ tracer technique compared net assimilation rates of fixed N₂ in shallow (5 m) and mesophotic (50 m) specimens of the scleractinian coral Stylophora pistillata, and the authors observed higher rates in the latter [11]. This difference was linked to an increased dependence on heterotrophy in mesophotic corals, however the choice of comparing shallow and mesophotic corals with clearly contrasting auto- vs. heterotrophic strategies may have masked the primary effect of depth-mediated light availability. Furthermore, the authors quantified depth-specific N₂ fixation rates in these corals only during one season, whereas a depth-specific seasonal response has not been investigated yet. In order to tease apart the effects of light and other seasonal factors in conspecifics with hypothetically similar nutritional strategies, we investigated N₂ fixation by the scleractinian coral S. pistillata along a shallower depth gradient.
Coral-associated N\textsubscript{2} fixation rates were quantified using the acetylene reduction assay in laboratory incubation experiments. In addition, gross photosynthesis rates ($P_g$) were measured in order to examine the respective autotrophic-heterotrophic status of the corals and to quantify the contribution of N\textsubscript{2} fixation to the corals' photo-metabolic N demand (CPND). We hypothesized similar seasonal responses in corals from all depths, with highest N\textsubscript{2} fixation rates during summer due to the lower environmental DIN concentrations during this season.

**Materials and methods**

**Coral collection and maintenance**

This study was conducted at a fringing coral reef located within a marine reserve in front of the Marine Science Station (MSS) at the northern Gulf of Aqaba (Red Sea), Jordan (29° 27'N, 34° 58'E). Permission for work within the marine reserve was issued by the University of Jordan and the MSS Aqaba. The fringing reef can be divided into a reef flat and a fore reef. Here, we focused on the fore reef, which consists of upper (4–8 m depth), middle (8–15 m depth) and lower (15–40 m depth) depth zones, each of which being characterized by distinctive 1) live coral cover, 2) coral species composition and 3) morphological features [30,31]. Live hard coral cover in the upper, middle and lower zone were approximately 15, 35 and 60%, respectively during the study period, with *S. pistillata* being abundant in each zone [2].

*S. pistillata* specimens (n = 7–8) were collected from individual colonies during two environmentally contrasting seasons, winter (02/03/2013) and summer (14/09/2013), by carefully chiseling fragments of similar size (5–6 cm in height, 1–2 cm diameter), morphology and pigmentation from the fore reef at 5, 10 and 20 m depth. To ensure biological replication as best as possible individual colonies were samples with a distance of at least 5 m in between. The distance between the individual sampling depth points along the gradual reef slope was approximately 50 m. Photosynthetically active radiation (PAR) and water temperature were measured seasonally at each depth using an underwater quantum sensor (LI-COR LI-192SA, Lincoln, Nebraska, USA) and HOBO loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: ± 0.53°C, Bourne, MA, USA), respectively and averaged from daily measurements conducted over seven consecutive days (mean ± SD; Fig 1A). On these days, temperature was recorded over 24 h in 1 min intervals, while PAR was recorded during the daily maximum from 12:00 to 13:00 in 1 min intervals. Further environmental data (i.e. water temperature, nutrient and Chl\textsubscript{a} concentrations) were retrieved from the Israel National Monitoring Program (http://www.iui-eilat.ac.il/Research/NMPMeteoData.aspx), in order to demonstrate changes in environmental conditions along a wider bathymetric gradient (0 to 600 m depth). For this analysis, an open-water monitoring station close (~6 km) to our study site was chosen, and data were compiled from the study period (March-September 2013; Fig 1B).

Coral specimens from each depth were individually glued with epoxy onto ceramic tiles and transferred to three inter-connected outdoor aquaria (800 L). Light intensities in the three aquaria were individually adjusted to comparative in situ light measurements at 5, 10 and 20 m depth, respectively, using variable layers of black mesh netting. Corals from each depth were placed in the aquarium with the depth-corresponding light intensity in order to avoid any change from in situ light levels. Adjusted light conditions (daily maximum) in the aquaria reached 350 and 450 $\mu$mol photons m\textsuperscript{-2} s\textsuperscript{-1} (5 m corals), 250 and 300 $\mu$mol photons m\textsuperscript{-2} s\textsuperscript{-1} (10 m corals) and 140 and 150 $\mu$mol photons m\textsuperscript{-2} s\textsuperscript{-1} (20 m corals) during winter and summer, respectively. The three aquaria were supplied with seawater freshly pumped from the reef at 10 m depth (exchange rate: 4000 L h\textsuperscript{-1}) ensuring that water temperature (23.0°C in winter and 27.8°C in summer) and other environmental parameters (i.e. nutrients) were comparable.
Fig 1. Environmental conditions at the study site. Environmental parameters (mean ± SD) measured at 5, 10 and 20 m depth at the study site (A) and along a 0–600 m depth gradient in the water column in the Gulf of Aqaba (B) during March 2013 (winter) and September 2013 (summer). Different lettering in panel A indicates significant differences for light levels (a–d) and water temperature (α–δ) between depths and seasons based on two-factor permutational ANOVAs with pairwise and Bonferroni corrected Monte Carlo tests (significance level, p < 0.05).

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between the light treatments. Corals were allowed to recover from fragmentation for 1 week before incubations were conducted in the aquaria under the depth-specific light conditions.

**Quantification of gross photosynthesis and N\textsubscript{2} fixation rates**

A detailed description of the chamber incubation procedure for quantifying \( P_g \) and coral-associated N\textsubscript{2} fixation can be found in Bednarz et al. (2015). Briefly, net photosynthesis (\( P_n \)) and respiration (\( R \)) rates were first quantified for all corals (\( n = 7–8 \) per depth and season) via \( O_2 \) flux measurements over 90 min in the light (light intensities were 350, 250, 140 μmol photons m\textsuperscript{-2} s\textsuperscript{-1} during winter and 450, 300, 150 μmol photons m\textsuperscript{-2} s\textsuperscript{-1} during summer for 5, 10 and 20-m corals, respectively) and in the dark (at night) with a conductivity- and temperature-corrected \( O_2 \) optode sensor (MultiLine\textsuperscript{R} IDS 3430, WTW GmbH, Weilheim, Bavaria, Germany, accuracy: ± 0.5% of measured value). All incubations were conducted in unfiltered seawater, and the \( O_2 \) concentration in the incubation chambers changed by ± 10% after 90 min. This \( O_2 \) difference was necessary to obtain measurable results without inducing stress in the corals [32]. \( O_2 \) fluxes by the corals were calculated by subtracting the initial \( O_2 \) concentrations from the final ones and correcting them with \( O_2 \) fluxes measured in seawater control (without corals) incubations. Then, \( O_2 \) fluxes were normalized to incubation time and the skeletal surface area of the corals. The skeletal surface area was measured using a standard geometric technique (Advanced Geometry) as described in [33]. Finally, \( P_g \) was calculated as \( P_g = P_n - R \). The total photosynthetic C acquisition (\( P_c \)) was calculated from \( P_g \) by converting \( O_2 \) fluxes into C equivalents based on molar weights and applying the formula \( P_c = \mu \text{mol C produced x 12}/PQ \), assuming a 12 h daylight period and a photosynthetic quotient (PQ) equal to 1.1 previously determined for \textit{S. pistillata} [34,35].

Following the \( O_2 \) flux measurements, N\textsubscript{2} fixation was measured on the same coral specimens using an adapted acetylene (\( C_2H_2 \)) reduction technique [36,37]. Corals were transferred without aerial exposure into 1-L chambers filled with 0.8 L of seawater. Additional chambers only filled with seawater served as controls. Immediately prior to the start of the incubations, 10% of the seawater was replaced by freshly produced \( C_2H_2 \)-saturated seawater. Chambers were then sealed ‘gastight’ before 10% of the headspace was replaced by freshly generated \( C_2H_2 \) gas. All chambers were positioned under the depth-specific light conditions and incubated under constant stirring (600 rpm) for a full dark-light cycle (24 h). Gas samples were drawn after 0 and 24 h and analyzed for ethylene (\( C_2H_4 \)) concentrations using a customized reducing compound photometer (Peak Laboratories, Mountain View, CA, USA, detection limit = 100 ppb). \( C_2H_4 \) evolution in each coral incubation chamber was seawater control corrected and calculated according to [38]. Finally, N\textsubscript{2} fixation rates were normalized to incubation time and the skeletal surface area of the corals. In order to estimate the CPND, measured acetylene reduction rates were converted into N equivalents using a conservative theoretical 4:1 (\( C_2H_4: N_2 \)) conversion ratio [36,39]. The photo-metabolic N demand was then calculated from the \( P_c \) rates assuming that only ~25% of the photosynthetically fixed C was incorporated into \textit{Symbiodinium} and host biomass (with the remaining fixed C assumed to be respired and released as organic C to the surrounding seawater as previously determined for \textit{S. pistillata} [40]) and assuming a C:N ratio of 7 [25]. Previously, a C:N ratio of 7 has been determined for \textit{Symbiodinium} of \textit{S. pistillata} corals collected from a 5 to 20 m depth gradient in the Gulf of Aqaba [25].

**Statistical analysis**

Data were analyzed using non-parametric permutational analysis of variance (PERMANOVA) in a univariate approach, since assumptions (i.e. normal distribution) for parametric analyses
were not met. Analyses were carried out using Primer-E version 6 software [41] with the PERMANOVA+ add on [42]. Two-factor PERMANOVAs were performed to test for differences in light availability, seawater temperature, \(N_2\) fixation, \(P_g\) and CPND between the three depths and the two seasons. Bray-Curtis similarities and type III (sequential) sum of squares were used for analyses with permutation of residuals under a reduced model (9999 permutations). The significance for the main test and for the pair-wise comparisons was based on Monte Carlo tests with Bonferroni corrected \(p\)-values to account for multiple comparisons (significance level, \(p < 0.05\)).

Results and discussion

Previous studies have described either seasonal or depth-specific differences in coral-associated \(N_2\) fixation rates, while the present study provides a comparison of seasonal differences across corals from different depths. The investigated environmental parameters (i.e. seawater temperature and light availability) varied differently across seasons and depths. Differences in water temperature (and likely also nutrient availability) were most pronounced on a seasonal scale than across the investigated depth range, whereas light levels decreased significantly from 5 to 20 m depth and varied seasonally only at shallower depths (Fig 1A). Overall, corals from all investigated depths showed active \(N_2\) fixation in both seasons, as indicated by higher \(C_2H_4\) concentrations measured in coral incubations compared to seawater controls. The acetylene reduction technique provides information about gross \(N_2\) fixation, rather than about the actual assimilation of fixed \(N_2\) by the coral. Here, we assume that ‘most’ of the \(N_2\) fixation-derived \(N\) was assimilated by the corals, since our \(N_2\) fixation rates (0.1–0.3 nmol \(C_2H_4\) cm\(^{-2}\) h\(^{-1}\) or 3.4–27.3 nmol N cm\(^{-2}\) d\(^{-1}\); Fig 2A) are in the same range as previously reported for \(S.\) \(pistillata\) from the Gulf of Aqaba using the \(^{15}N_2\) tracer technique [11]. Similar \(N_2\) fixation rates in \(S.\) \(pistillata\) colonies were also reported from the Great Barrier Reef [29], while conspecifics from New Caledonia showed 10-times higher rates [43]. Besides measurement and technique-associated differences, such geographic variations may also suggest that certain locations are characterized by environmental conditions that stimulate the abundance and/or activity of coral-associated diazotrophs. However, it is still under debate whether diazotroph-derived \(N\) is actually translocated from the bacteria to the coral-algae symbiosis. Thus, localizing and tracing the fate of this \(N\) within different cells of the coral holobiont will be required to ultimately understand the role of diazotrophs in coral nutrition.

In the present study, \(N_2\) fixation was found to differ significantly across seasons, although this seasonal effect only occurred for corals from shallower (5 and 10 m) depths (Table 1 and Fig 2A). These corals from 5 and 10 m depths (hereafter referred to as “shallow corals”) were characterized by statistically significant, 6-fold higher rates of \(N_2\) fixation in summer as compared to winter. By contrast, corals from 20 m (hereafter referred to as “deep corals”) fixed \(N_2\) at similar rates in both seasons. \(P_g\) rates of corals from all depths were similar within each season (Fig 2B), demonstrating that the seasonal variability in \(N_2\) fixation rates is independent of the coral’s autotrophic status (at least in colonies of the depths surveyed).

The annual stratification cycle in the Gulf of Aqaba results in pronounced seasonal fluctuations in environmental parameters, such as water temperature and nutrient levels [19,44]. In summer the formation of a nutricline at ~100 m depth causes nutrient depletion in the stratified upper water column, while deep-water mixing during winter brings nutrient-rich seawater back into the reef zone (Fig 1B) [20,23]. Elevated DIN availability can inhibit the energy-costly process of \(N_2\) fixation in favor of DIN assimilation [45], whereas the more pronounced oligotrophic conditions in summer favor coral-associated \(N_2\) fixation [12,21]. Also, the abundance of potential diazotrophic bacteria associated with corals increases during seasons with reduced

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Coral-associated \(N_2\) fixation at different water depths

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Fig 2. \(\text{N}_2\) fixation and photosynthesis rates in \textit{Stylophora pistillata}. \(\text{N}_2\) fixation expressed as ethylene production or amount of nitrogen fixed (A), gross photosynthesis expressed as \(P_g\) or \(P_c\) (B) and the contribution of fixed nitrogen to the photo-metabolic nitrogen demand (C) in \textit{Stylophora pistillata} corals. All rates were quantified in corals collected from three different depths (5, 10 and 20 m) during winter and summer (\(n = 7-8\); mean ± SE). Different lettering (a-c) indicates significant differences between depths and seasons based on two-factor permutational ANOVAs with pairwise and Bonferroni corrected Monte Carlo tests (significance level, \(p < 0.05\)).

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DIN availability in the seawater [46], indicating that corals might be able to acquire some additional N from these bacteria. In the present study, the CPND increased significantly from 1–4% during winter to 7–11% during summer (Fig 2C), suggesting that N$_2$ fixation may compensate for the reduced DIN availability during summer by contributing more N to the N budget of 

$S$. pistillata. Since 

$S$. pistillata colonies of the Gulf of Aqaba normally experience decreased 

$Symbiodinium$ densities, alongside increased Chl $a$ content per 

$Symbiodinium$ cell, during summer [12], N$_2$ fixation-derived N may be relatively more important for coral productivity in this season; this fixed and presumable translocated N might also be important in re-establishing peak 

$Symbiodinium$ densities at the end of the summer season [47,3]. Interestingly, only the shallow corals showed increased N$_2$ fixation rates during summer, which is in line with previous seasonal observations on scleractinian and soft corals from 10 m depth in the Gulf of Aqaba [12,21]. In contrast, N$_2$ fixation rates in deep corals were seasonally stable. Consequently, the CPND was seasonally stable in deep corals, while it significantly increased in shallow-water from winter to summer. This depth-specific seasonal response of coral-associated N$_2$ fixation cannot be directly explained by the seasonally variable DIN availability, since DIN concentrations within each season were similar across the investigated depth range (5–20 m) (Fig 1). Rather, depth-driven light differences that alter coral physiology (e. g.,

### Table 1. Statistical results for differences in environmental conditions and coral-associated physiological parameters between depths and seasons.

| Variables | Effect | df | SS   | MS   | Pseudo F | p (MC) | Fig |
|-----------|--------|----|------|------|----------|--------|-----|
| PAR (μmol photons m$^{-2}$ s$^{-1}$) | Depth (De) | 2  | 85.48 | 42.74 | 244.84   | <0.001 | 1A  |
|          | Season (Se) | 1  | 5.31  | 5.31  | 30.41    | <0.001 |     |
|          | De x Se    | 2  | 3.36  | 1.68  | 9.62     | <0.001 |     |
|          | Residuals  | 108| 18.85 | 0.17  |          |        |     |
|          | Total      | 113| 113   |       |          |        |     |
| Seawater temperature (°C) | Depth (De) | 2  | 1.59  | 0.79  | 60.18    | <0.001 | 1A  |
|          | Season (Se) | 1  | 109.94 | 109.94 | 8336.60 | <0.001 |     |
|          | De x Se    | 2  | 0.05  | 0.02  | 1.83     | 0.165  |     |
|          | Residuals  | 108| 1.42  | 0.01  |          |        |     |
|          | Total      | 113| 113   |       |          |        |     |
| N$_2$ fixation (nmol C$_2$H$_4$ cm$^{-2}$ h$^{-1}$ or nmol N cm$^{-2}$ d$^{-1}$) | Depth (De) | 2  | 5514  | 2757  | 3.205    | 0.011  | 2A  |
|          | Season (Se) | 1  | 17351 | 17351 | 20.171   | <0.001 |     |
|          | De x Se    | 2  | 9047  | 4524  | 5.2587   | <0.001 |     |
|          | Residuals  | 38  | 32688 | 860   |          |        |     |
|          | Total      | 43  | 66121 |       |          |        |     |
| Gross photosynthesis (μmol O$_2$ cm$^{-2}$ h$^{-1}$ or μmol C cm$^{-2}$ d$^{-1}$) | Depth (De) | 2  | 392   | 196   | 1.176    | 0.318  | 2B  |
|          | Season (Se) | 1  | 4199  | 4199  | 25.209   | <0.001 |     |
|          | De x Se    | 2  | 118   | 59    | 0.356    | 0.764  |     |
|          | Residuals  | 38  | 6329  | 167   |          |        |     |
|          | Total      | 43  | 11076 |       |          |        |     |
| CPND (%) | Depth (De) | 2  | 7160  | 3580  | 3.893    | 0.004  | 2C  |
|          | Season (Se) | 1  | 26171 | 26171 | 28.641   | <0.001 |     |
|          | De x Se    | 2  | 8445  | 4222  | 4.592    | <0.001 |     |
|          | Residuals  | 38  | 34943 | 920   |          |        |     |
|          | Total      | 43  | 78386 |       |          |        |     |

Results of the two-factorial permutational ANOVAs for testing the effects of depth (5, 10 and 20 m) and season (winter and summer) on photosynthetically active radiation (PAR) and seawater temperature during the study period as well as on N$_2$ fixation, gross photosynthesis and on the contribution of N$_2$ fixation to the corals' photo-metabolic nitrogen demand (CPND) in 

$S$. pistillata. Statistically significant Monte Carlo (MC) p-values (<0.05) are highlighted in bold.

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mucus release by the coral host and nutrient acquisition by the in hospite *Symbiodinium* community) and/or variations in the diazotrophic community of the coral holobionts across depths may instead have affected N$_2$ fixation rates.

The photosynthetic efficiency of *Symbiodinium* increases with light availability and correlates positively with the amount of C translocated to the coral host [48,49]. Thus, under higher light availability the coral host receives more C than required for its metabolism and releases the excess C as organic matter to the surrounding seawater [50]. Heterotrophic diazotrophs in particular depend on energy-rich organic matter that is assimilated from the surrounding seawater and/or provided by the coral host [51]. The coral mucus surface layer with its high organic C content [52,53] represents a suitable habitat for heterotrophic bacteria and contains high abundances of active diazotrophs [7,11,54]. Since depth- and seasonal-driven light differences change the quality and quantity of coral-derived mucus, they may consequently affect coral-associated N$_2$ fixation. Indeed, total organic matter (i.e. mucus) release by shallow *S. pistillata* corals significantly increases during the summer season at the same study location [12], likely as a result of elevated light availability [55,56]. This would provide heterotrophic diazotrophs with an energy-rich food source and may explain the observed increased N$_2$ fixation activity in shallow corals during summer. In contrast to their shallow-water conspecifics, deep-water corals are likely to release organic matter at consistent rates throughout the year due to seasonally less variable light availabilities, and this may account for the seasonally stable N$_2$ fixation rates observed herein.

Besides light- and photosynthesis-driven changes in coral mucus release rates, the coral-associated diazotrophic communities themselves can also change along depth gradients, and this may have also contributed to the depth-related variation in N$_2$ fixation rates. A recent study found significant differences in the diazotrophic community of *S. pistillata* colonies collected from 5 and 15 m depths on the Great Barrier Reef [29]. Interestingly, variations in light exposure significantly changed the community associated with 5 m corals, while no light effect was found for the community associated with 15 m corals. Since the diazotrophic community composition of shallow corals seems to show a more pronounced response to changes in light levels [29], light may also have a stronger effect on the activity of these bacteria. In the present study, shallow corals experienced a more pronounced seasonal change in light availability compared to the deep-water corals; such light level variation may have been associated with greater seasonal changes in the diazotrophic community, as well as diazotroph cell densities, and, therefore, resulted in the seasonally more variable N$_2$ fixation rates in these shallow-water corals.

Besides changes in the diazotrophic community, the dominant coral-associated *Symbiodinium* genotype can also vary along depth gradients with certain *Symbiodinium* types (clades) being more efficient at photosynthesizing and assimilating nutrients than others in hospite [48,57,58]. This can lead to differing levels of photosynthetic and nutrient transfer to the coral host [48,57,58] and may subsequently influence the coral’s response to seasonally changing environmental conditions (e.g. DIN availability). In the northern Red Sea, *S. pistillata* corals shift from hosting *Symbiodinium* clade A in shallow depths (5–10 m) to clade C below 40 m depth [24,27]. At intermediate depths of 20 m, *S. pistillata* starts to primarily host clade C over clade A [59]. The DIN assimilation capacity of clade A is ~10-times higher than for clade C, suggesting that shallow corals are able to utilize the increased DIN available during winter more efficiently than corals hosting clade C [58,60]. Consequently, shallow corals are likely to be less dependent on diazotrophically-derived N during winter, which may cause the significant drop in N$_2$ fixation rates. Although speculative at this time, a physiological linkage between *Symbiodinium* genotype and N$_2$ fixation may exist, since *Symbiodinium* can host their own diazotrophic community [61] and are the primary site for diazotrophically-derived N
uptake within the coral symbiosis [28,43]. In future experiments, we recommend using corals experimentally infected with different Symbiodinium clades, such that the specific effect of host and Symbiodinium genotype on N₂ fixation can be tested.

In conclusion, the results presented in this study indicate that, rather than a gradual change in coral-associated N₂ fixation along the depth gradient, there is instead a division into two vertically distributed groups: 1) seasonally variable N₂ fixation in shallow-water corals (0–15 m depth) and 2) seasonally stable N₂ fixation in deep-water corals (20 m depth). In future experiments, it will be interesting to determine if the 1) activity, 2) abundance and 3) community composition of coral-associated diazotrophs also show a depth-specific response to globally changing environmental conditions, as well as whether any corresponding differences in N₂ fixation activity have the potential to differentially influence the resilience and/or stress response of coral holobionts to climate change.

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References
1. Rohwer F, Seguritan V, Azam F, Knowlton N. Diversity and distribution of coral-associated bacteria. Mar Ecol Prog Ser. 2002; 243: 1–10.
2. Cardini U, Bednarz VN, van Hoytema N, Rovere A, Naumann MS, Al-Rshaidat MMD, et al. Budget of primary production and dinitrogen fixation in a highly seasonal Red Sea coral reef. Ecosystems. 2016; 19: 771–785.
3. Dubinsky Z, Falkowski P. Light as a source of information and energy in zooxanthellate corals. Coral Reefs: An Ecosystem in Transition. Dordrecht: Springer Netherlands; 2011. pp. 107–118.
4. Wang TJ, Douglas EA. Essential amino acid synthesis and nitrogen recycling in an alga-invertebrate symbiosis. Mar Biol. 1999; 135: 219–222.
5. Tanaka Y, Grottoli AG, Matsui Y, Suzuki A, Sakai K. Partitioning of nitrogen sources to algal endosymbionts of corals with long-term ¹⁵N-labelling and a mixing model. Ecol Modell. 2015; 309–310: 163–169.
6. Rahav O, Dubinsky Z, Achituv Y, Falkowski PG. Ammonium metabolism in the zooxanthellate coral, Stylophora pistillata. Proc R Soc B Biol Sci. 1988; 236: 325–337.
7. Lema K, Willis B, Bourne D. Corals form characteristic associations with symbiotic nitrogen-fixing bacteria. Appl Environ Microbiol. 2012; 78: 3136–3144. https://doi.org/10.1128/AEM.07800-11 PMID: 22344646
8. Benavides M, Bednarz VN, Ferrier-Pagès C. Diazotrophs: Overlooked key players within the coral symbiosis and tropical reef ecosystems? Front Mar Sci. 2017; 4: 10.
9. Shashar N, Cohen Y, Loya Y, Sar N. Nitrogen fixation (acetylene reduction) in stony corals: evidence for coral-bacteria interactions. Mar Ecol Prog Ser. 1994; 111: 259–264.

10. Lesser MP, Mazel CH, Gorbunov MY, Falkowski PG. Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. Science 2004; 305: 997–1000. https://doi.org/10.1126/science.1099128 PMID: 15310901

11. Bednarz VN, Grover R, Maguer J-F, Fine M, Ferrier-Pagès C. The assimilation of diazotroph-derived nitrogen by scleractinian corals depends on their metabolic status. MBio. 2017; 8: e02058–16. https://doi.org/10.1128/mBio.02058-16 PMID: 28074021

12. Cardini U, Bednarz VN, Naumann MS, van Hoytema N, Rix L, Foster RA, et al. Functional significance of dinitrogen fixation in sustaining coral productivity under oligotrophic conditions. Proc R Soc B Biol Sci. 2015; 282: 20152257.

13. Cardini U, Bednarz VN, Foster RA, Wild C. Benthic N$_2$ fixation in coral reefs and the potential effects of human-induced environmental change. Ecol Evol. 2014; 4: 1706–1727. https://doi.org/10.1002/ece3.1050 PMID: 24967086

14. Cardini U, van Hoytema N, Bednarz VN, Rix L, Foster RA, Al-Rshaidat MMD, et al. Microbial dinitrogen fixation in coral holobionts exposed to thermal stress and bleaching. Environ Microbiol. 2016; 18: 2620–2633. https://doi.org/10.1111/1462-2920.13385 PMID: 27234003

15. Breitbarth E, Oschlies A, LaRoche J. Physiological constraints on the global distribution of *Trichodesmium*? effect of temperature on diazotrophy. Biogeoosciences. 2007; 4: 53–61.

16. Gallon JR. N$_2$ fixation in phototrophs: adaptation to a specialized way of life. Plant Soil. 2001; 230: 39–48.

17. Charpy L, Alliod R, Rodier M, Golubic S. Benthic nitrogen fixation in the SW New Caledonia lagoon. Aquat Microb Ecol. 2007; 47: 73–81.

18. Gallon JR. Reconciling the incompatible: N$_2$ fixation and O$_2$. New Phytol. Blackwell Publishing Ltd; 1992; 122: 571–609.

19. Carlson DF, Fredj E, Gildor H. The annual cycle of vertical mixing and restratification in the Northern Gulf of Elat/Aqaba (Red Sea) based on high temporal and vertical resolution observations. Deep Sea Res Part I Oceanogr Res Pap. 2014; 84: 1–17.

20. Manasrah R, Raheed M, Badran MI. Relationships between water temperature, nutrients and dissolved oxygen in the northern Gulf of Aqaba, Red Sea. Oceanologia. 2006; 48: 237–253.

21. Bednarz VN, Cardini U, van Hoytema N, Al-Rshaidat MMD, Wild C. Seasonal variation in dinitrogen fixation and oxygen fluxes associated with two dominant zooxanthellate soft corals from the northern Red Sea. Mar Ecol Prog Ser. 2015; 519: 141–152.

22. Paulson CA, Simpson JJ. Irradiance measurements in the upper ocean. J Phys Oceanogr. 1977; 7: 952–956.

23. Rasheed M, Badran MI, Huettel M. Particulate matter filtration and seasonal nutrient dynamics in permeable carbonate and silicate sands of the Gulf of Aqaba, Red Sea. Coral Reefs. 2003; 22: 167–177.

24. Mass T, Einbinder S, Brokovich E, Shashar N, Vago R, Erez J, et al. Photoacclimation of *Stylophora pistillata* to light extremes: metabolism and calcification. Mar Ecol Prog Ser. 2007; 334: 93–102.

25. Alamaru A, Loya Y, Brokovich E, Yam R, Shemesh A. Carbon and nitrogen utilization in two species of Red Sea corals along a depth gradient: Insights from stable isotope analysis of total organic material and lipids. Geochim Cosmochim Acta. 2009; 73: 5333–5342.

26. Lesser MP, Slattery M, Stat M, Ojimi M, Gates RD, Grottoli A. Photoacclimatization by the coral *Montastraea cavernosa* in the mesophotic zone: light, food, and genetics. Ecology. 2010; 91: 990–1003. PMID: 20462114

27. Winters G, Beer S, Zvi B, Brickner I, Loya Y. Spatial and temporal photoacclimation of *Stylophora pistillata*: zooxanthella size, pigmentation, location and clade. Mar Ecol Prog Ser. 2009; 384: 107–119.

28. Lesser M, Falcón L, Rodríguez-Román A, Enríquez S, Hoegh-Guldberg O, Iglesias-Prieto R. Nitrogen fixation by symbiotic cyanobacteria provides a source of nitrogen for the scleractinian coral *Montastraea cavernosa*. Mar Ecol Prog Ser. 2007; 346: 143–152.

29. Lesser MP, Morrow KM, Pankey SM, Noonan SHC. Diazotroph diversity and nitrogen fixation in the coral *Stylophora pistillata* from the Great Barrier Reef. ISME J. 2017; 1. https://doi.org/10.1038/s41396-017-0008-6 PMID: 29222444

30. Mergner H, Schuhmacher H. Morphologie, Ökologie und Zonierung von Korallenriffen bei Aqaba, (Golf von Aqaba, Rotes Meer). Helgoländer Wissenschaftliche Meeresuntersuchungen. 1974; 26: 238–358.

31. Naumann MS, Richter C, Mott C, el-Zibdah M, Manasrah R, Wild C. Budget of coral-derived organic carbon in a fringing coral reef of the Gulf of Aqaba, Red Sea. J Mar Syst. 2012; 105–108: 20–29.
32. Haas AF, Smith JE, Thompson M, Deheyn DD. Effects of reduced dissolved oxygen concentrations on physiology and fluorescence of hermatypic corals and benthic algae. PeerJ. 2014; 2: e235. https://doi.org/10.7717/peerj.235 PMID: 24482757

33. Naumann MS, Niggl W, Laforsch C, Glaser C, Wild C. Coral surface area quantification–evaluation of established techniques by comparison with computer tomography. Coral Reefs. 2009; 28: 109–117.

34. Muscatine L, R. McCloskey L, E. Marian R. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration1. Limnol Oceanogr. 1981; 26: 601–611.

35. Gattuso J-P, Jaubert J. Effect of light on oxygen and carbon dioxide fluxes and on metabolic quotients measured in situ in a zooxanthellate coral. Limnol Oceanogr. 1990; 35: 1796–1804.

36. Capone DG. Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. Handbook of methods in aquatic microbial ecology. 1993. pp. 621–631.

37. Wilson ST, Böttjer D, Church MJ, Karl DM. Comparative assessment of nitrogen fixation methodologies, conducted in the oligotrophic North Pacific Ocean. Appl Environ Microbiol. 2012; 78: 6516–23. https://doi.org/10.1128/AEM.01146-12 PMID: 22773638

38. Breitbarth E, Mills MM, Friedrichs G, LaRoche J. The Bunsen gas solubility coefficient of ethylene as a function of temperature and salinity and its importance for nitrogen fixation assays. Limnol Oceanogr Methods. 2004; 2: 282–288.

39. Mulholland MR, Bronk DA, Capone DG. Dinitrogen fixation and release of ammonium and dissolved organic nitrogen by Trichodesmium IMS101. Aquat Microb Ecol. 2004; 37: 85–94.

40. Tremblay P, Grover R, Maguer JF, Hoogenboom M, Ferrier-Pagès C. Carbon translocation from symbiont to host depends on irradiance and food availability in the tropical coral Stylophora pistillata. Coral Reefs. 2014; 33: 1–13.

41. Clarke KR, Gorley RN. Primer version 6: user manual/tutorial Primer-E. Plymouth, England: 2006.

42. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 2001; 26: 32–46.

43. Benavides M, Houlbrèque F, Camps M, Lorrain A, Grosso O, Bonnet S. Diazotrophs: a non-negligible source of nitrogen for the tropical coral Stylophora pistillata. J Exp Biol. 2016; 219: 2608–2612. https://doi.org/10.1242/jeb.139451 PMID: 27335448

44. Silverman J, Lazar B, Erez J. Community metabolism of a coral reef exposed to naturally varying dissolved inorganic nutrient loads. Biogeochemistry. 2007; 84: 67–82.

45. Falkowski PG. Enzymology of nitrogen assimilation. In: Carpenter EJ, Capone DG, editors. Nitrogen in the Marine Environment. New York: Academic Press; 1983. pp. 839–868.

46. Chen C-P, Tseng C-H, Chen CA, Tang S-L. The dynamics of microbial partnerships in the coral Isopora palifera. ISME J. 2011; 5: 728–740. https://doi.org/10.1038/ismej.2010.151 PMID: 20962876

47. Dubinsky Z, Jokiel P. Ratio of energy and nutrient fluxes regulates symbiosis between zooxanthellae and corals. Pacific Sci. 1994; 48: 313–324.

48. Leal MC, Hoadley K, Pettay DT, Grajales A, Calado R, Warner ME. Symbiont type influences trophic plasticity of a model cnidarian–dinoflagellate symbiosis. J Exp Biol. 2015; 218: 858–863. https://doi.org/10.1242/jeb.115519 PMID: 25617454

49. Tremblay P, Maguer JF, Grover R, Ferrier-Pagès C. Trophic dynamics of scleractinian corals: stable isotope evidence. J Exp Biol. 2015; 218: 1223–1234. https://doi.org/10.1242/jeb.115303 PMID: 25722004

50. Tremblay P, Ferrier-Pagès C, Maguer JF, Rottier C, Legendre L, Grover R. Controlling effects of irradiance and heterotrophy on carbon translocation in the temperate coral Cladocora caespitosa. PLoS One. 2012; 7: e44672. https://doi.org/10.1371/journal.pone.0044672 PMID: 22970284

51. Allers E, Nieser C, Wild C, Penthaler J. Microbes enriched in seawater after addition of coral mucus. Appl Environ Microbiol. 2008; 74: 3274–3278. https://doi.org/10.1128/AEM.01870-07 PMID: 18344335

52. Wild C, Woyt H, Huettel M. Influence of coral mucus on nutrient fluxes in carbonate sands. Mar Ecol Prog Ser. 2005; 287: 87–98.

53. Bythell JC, Wild C. Biology and ecology of coral mucus release. J Exp Mar Bio Ecol. 2011; 408: 88–93.

54. Camps M, Benavides M, Lema K, Boume D, Grosso O, Bonnet S. Released coral mucus does not enhance planktonic N2 fixation rates. Aquat Microb Ecol. 2016; 77: 51–63.

55. Crossland C. In situ release of mucus and DOC-lipid from the corals Acropora variabilis and Stylophora pistillata in different light regimes. Coral Reefs. 1987; 6: 35–42.

56. Naumann MS, Haas A, Struck U, Mayr C, el-Zibdah M, Wild C. Organic matter release by dominant hermatypic corals of the Northern Red Sea. Coral Reefs. 2010; 29: 649–659.
57. Pernice M, Dunn SR, Tonk L, Dove S, Domart-Coulon I, Hoppe P, et al. A nanoscale secondary ion mass spectrometry study of dinoflagellate functional diversity in reef-building corals. Environ Microbiol. 2015; 17: 3570–3580. https://doi.org/10.1111/1462-2920.12518 PMID: 24902979

58. Ezzat L, Fine M, Maguer J-F, Grover R, Ferrier-Pagès C. Carbon and nitrogen acquisition in shallow and deep holobionts of the scleractinian coral *S. pistillata*. Front Mar Sci. Frontiers; 2017; 4: 102.

59. Lampert-Karako S, Stambler N, Katcoff DJ, Achituv Y, Dubinsky Z, Simon-Blecher N. Effects of depth and eutrophication on the zooxanthellae clades of *Stylophora pistillata* from the Gulf of Eilat (Red Sea). Aquat Conserv Mar Freshw Ecosyst. 2008; 18: 1039–1045.

60. Baker DM, Andras JP, Jordán-Garza AG, Fogel ML. Nitrate competition in a coral symbiosis varies with temperature among *Symbiodinium* clades. ISME J. 2013; 7: 1248–1251. https://doi.org/10.1038/ismej.2013.12 PMID: 23407311

61. Ainsworth TD, Krause L, Bridge T, Torda G, Raina J-B, Zakrzewski M, et al. The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. ISME J. 2015; 9: 2261–2274. https://doi.org/10.1038/ismej.2015.39 PMID: 25885563