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Sugar enrichment provides evidence for a role of nitrogen fixation in coral bleaching

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Abstract

The disruption of the coral–algae symbiosis (coral bleaching) due to rising sea surface temperatures has become an unprecedented global threat to coral reefs. Despite decades of research, our ability to manage mass bleaching events remains hampered by an incomplete mechanistic understanding of the processes involved. In this study, we induced a coral bleaching phenotype in the absence of heat and light stress by adding sugars. The sugar addition resulted in coral symbiotic breakdown accompanied by a fourfold increase of coral-associated microbial nitrogen fixation. Concomitantly, increased N:P ratios by the coral host and algal symbionts suggest excess availability of nitrogen and a disruption of the nitrogen limitation within the coral holobiont. As nitrogen fixation is similarly stimulated in ocean warming scenarios, here we propose a refined coral bleaching model integrating the cascading effects of stimulated microbial nitrogen fixation. This model highlights the putative role of nitrogen-fixing microbes in coral holobiont functioning and breakdown.

KEYWORDS
coral reefs, diazotroph, microbial activity, nitrogen cycling, *Pocillopora*, *Symbiodinium*, symbiosis

1 | INTRODUCTION

The symbiosis between reef-building corals and dinoflagellate algae of the genus *Symbiodinium* provides the foundation for the ecological success of coral reefs over millions of years (Muscatine & Porter, 1977). In this mutualistic association, the coral host provides inorganic nutrients in exchange for photosynthetically fixed carbon (photosynthates) and amino acids from the algal symbiont (Muscatine & Porter, 1977). Coral bleaching, the disruption of this delicate symbiosis by heat and light stress or poor water quality (Fabricius, 2005; Lesser, 1996; Wooldridge & Done, 2009), may ultimately result in the mortality of the coral host. Mass bleaching events have resulted in unprecedented degradation of coral reefs over the past decades and are expected to increase in frequency

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and severity as global climate change progresses (Hughes et al., 2003).

Even though several decades have passed since the initial observation of large-scale coral bleaching, our understanding of the underlying mechanistic processes remains incomplete. Among the proposed mechanisms, particularly the idea of oxidative stress as a driver of coral bleaching (Oxidative Theory of Bleaching) has found considerable resonance (Downs et al., 2002). This theory posits that the bleaching cascade is initiated by oxidative stress in the algal symbionts (and host tissues) caused by excessive temperature and light conditions (Lesser, 1996). Yet, there is emerging evidence for a more complex mechanistic response, intimately linking bleaching with environmental nitrogen (N) availability (Vega Thurber et al., 2014; Wiedenmann et al., 2012; Wooldridge, 2013; Wooldridge & Done, 2009). As N limitation is required to regulate *Symbiodinium* cell division rates and to promote the translocation of photosynthates to the coral, N enrichment threatens the persistence of this symbiosis (Dubinsky & Jokiel, 1994; Falkowski, Dubinsky, Muscatine, & McCloskey, 1993). Specifically, it can reduce photosynthate translocation rates by *Symbiodinium* (Suescún-Bolívar, Traverse, & Thomé, 2016). Accordingly, Wooldridge (2013) proposed that this retention of photosynthates would result in the energy limitation of coral carbon concentrating mechanisms (CCMs). The resulting carbon dioxide (CO_2) limitation of photosynthetic "dark reactions" would render *Symbiodinium* more susceptible to photodamage (i.e., bleaching). Indeed, the idea of the "selfish symbiont" was recently confirmed by Ezzat, Maguer, Grover, and Ferrier-Pagès (2015), who reported an increased utilization and reduced translocation rates of photosynthetically fixed carbon by *Symbiodinium in hospite* under nutrient replete growth scenarios. Further, excess N availability can lower the bleaching threshold in corals by shifting *Symbiodinium* from an N limited to a phosphorus (P)-starved state (Wiedenmann et al., 2012). Such stoichiometric shifts can cause the substitution of phospholipids with sulpholipids in the chloroplast thylakoid membranes, a common response in photoautotrophs during limited P availability (Frentzen, 2004). As the lipid composition of the thylakoid membrane is closely linked to the assemblage and functioning of the photosynthetic machinery, it can determine bleaching sensitivity in *Symbiodinium* (Tchernov et al., 2004). Therefore, increased N availability will ultimately increase the bleaching susceptibility of corals (Wiedenmann et al., 2012).

Our understanding of internal N cycling processes in corals during thermal bleaching remains incomplete. This knowledge, however, is critical, as N cycling microbes are ubiquitous associates of corals (Rädecker, Pogoreutz, Voolstra, Wiedenmann, & Wild, 2015). In particular, diazotrophs, i.e. Bacteria and Archaea capable of reducing dinitrogen (N_2) into biologically available N, constitute an important N source for *Symbiodinium* (Bednarz, Grover Maguer, Fine, & Ferrier-Pagès, 2017; Benavides et al., 2016; Lema, Willis, & Bourne, 2012; Lema et al., 2016; Lesser et al., 2007). Indeed, N_2 fixation can help sustain coral holobiont productivity when nutrients are scarce (Cardini et al., 2015). Given its functional importance, it is not surprising that N_2 fixation is associated with the majority of investigated coral species (Cardini et al., 2015; Rädecker et al., 2014; Shashar, Cohen, Loya, & Sar, 1994). Elevated temperatures, however, stimulate the enzymatic activity of nitrogenase and promote the proliferation and activity of coral-associated diazotrophs (Cardini et al., 2015; Cardini et al., 2016; Compaoré & Stal, 2010; Santos et al., 2014). Consequently, this has led Rädecker et al. (2015) to propose that excess N availability from increased holobiont-associated N_2 fixation activity may be a major driver of bleaching in heat-stressed corals.

Here, we aimed to gain a better mechanistic understanding of the role of N_2 fixing Bacteria and Archaea during coral bleaching. For this purpose, we manipulatively stimulated N_2 fixation activity in corals in the absence of heat or light stress. To achieve this, we supplied doses of labile dissolved organic carbon (DOC), more specifically neutral monosaccharides, to stimulate coral-associated N_2 fixation (Shashar et al., 1994). This approach allowed us to identify the effects of increased N_2 fixation activity on the coral–algal symbiosis, while eliminating the confounding effects of temperature and irradiance. We characterized the cascading effects on critical functions of the coral holobiont and three of its main members—the coral host, algal symbionts, and the prokaryotes—in an integrative approach combining physiological and molecular applications.

2 | MATERIALS AND METHODS

2.1 | Aquarium facilities, coral collection, and maintenance

The experiments were conducted at the wet laboratory facility of the Coastal and Marine Resources Core Lab (CMOR) at the King Abdullah University of Science and Technology (KAUST, KSA). The aquarium system was comprised of two identical units, each consisting of three replicate experimental tanks (i.e., totaling six tanks 100 L each). To stabilize seawater parameters and oxygen (O_2) concentrations, untreated Red Sea reef water was circulated in the experimental units, each containing protein skimmer as well as filtration setups. Further, 30% of the water was replaced on a daily basis, assuring close to natural water parameters. Maintenance conditions were kept constant, allowing us to rear corals in the absence of any heat or light stress (seawater temperature at 27°C, salinity at 40.5 PSU, photosynthetic active radiation 100 quanta $\mu mol\ s^{-1}\ m^{-2}$ on a 12:12-hr day/light cycle). In three aquaria, labile DOC levels were manipulated by daily additions of a 10 mg/L saccharide mixture (in mg/L; (D+) xylose: 3.82; (D+) glucose: 2.56; (D+) mannose: 1.39; (D+) galactose: 2.22). Respective contribution of each saccharide was based on reports on the neutral monosaccharide composition of sewage and coral reef macroalgae exudates (Huang, Li, & Gu, 2010; Nelson et al., 2013). The other three aquaria were maintained at ambient DOC levels. To avoid drifting effects on the labile DOC concentrations across the replicate tanks, they were supplied from a recirculation reservoir (100 L) according to treatment conditions. The DOC treatment resulted in >10 times enriched conditions (up to 1609 ± 2 μM after 28 days of treatment) compared to the ambient treatment (117 ± 2 μM after

28 days; Table S1). The enrichment did not affect dissolved O_2 levels in the treatment tanks (constantly >6 mg/L) or total N and total P concentrations at any time point; for details, see Tables S1 and S2).

Six colonies of the common Red Sea coral *Pocillopora verrucosa* were collected at the mid-shore reef Al-Fahal in the Central Red Sea, Saudi Arabia (N22°18′19.98″, E38°57′46.08″). Each colony was fragmented, and the fragments attached to 40 × 40 mm stone tiles with a two-part epoxy putty (ReefConstruct, AquaMedic, Germany). Coral fragments from all colonies were distributed evenly among aquaria tanks and acclimated for 28 days. During this acclimation period, corals were moderately fed to assist recovery from fragmentation stress (Reef Roids, PolypLab, USA). Any additional feeding was abandoned 1 week prior to and throughout the experiment to avoid confounding effects from additional nutrient uptake via heterotrophy.

2.2 | Sampling

N_2 fixation activity, diazotroph abundance, maximum quantum yield, and *Symbiodinium* density were measured at days 0, 7, 12, and 28 of the experiment. Remaining response parameters were measured at the first and last day of the experiment. Noninvasive parameters (pulse amplitude fluorometry, rate functions) were applied in a repeated measures design to increase statistical power. For the remaining (invasive) response parameters, single fragments originating from all mother colonies and treatments were rinsed with filter-sterilized seawater (FSW; 0.22 μm), flash-frozen in liquid N_2, and stored at −80°C until further processing. Seawater samples were collected every 7 days, filtered, and frozen for subsequent analysis of DOC, total dissolved N (TN), and P (TP) content. A brief overview of all measured response parameters is provided in the following; please refer to Supplementary Methods for a more detailed description.

2.3 | O_2 and N_2 fixation measurements

Photosynthesis and respiration rates were derived from O_2 evolution/depletion incubations. For this, net photosynthesis and respirations rates were quantified from start and endpoint O_2 measurements of corals incubated in gastight chambers for 2 hr during dark and light conditions, respectively (Rädecker et al., 2014). Gross photosynthesis rates (P_G) were calculated as the combination of net photosynthesis (P_N) and respiration rates (R). Similarly, gross N_2 fixation rates were indirectly quantified via measurements of ethylene (C_2H_4) evolution using the acetylene (C_2H_2) reduction assay (Wilson et al., 2012). Specifically, corals were incubated for 24 hr in gastight chambers containing seawater as well as an air-filled headspace both enriched in C_2H_2. N_2 fixation rates were inferred from differences in C_2H_4 concentrations of gas samples collected at the start and end of the light as well as the dark phase of incubation.

2.4 | *Symbiodinium* response and elemental analyses

Photosynthetic performance of *Symbiodinium* cells *in hospite* was confirmed by measuring PSII maximum quantum yield (F_v/F_m) of dark-adapted coral fragments (n = 12 per treatment) 1 hr into the 12-hr dark phase. Measurements were carried out using a pulse amplitude modulation (PAM) fluorometer (DIVING-PAM, Walz, Germany). To assess symbiont density, *Symbiodinium* cells were freshly isolated from coral tissue by NaOH extraction (Zamoum & Furla, 2012). *Symbiodinium* cell counts were determined using flow cytometry and normalized to coral fragment surface areas (Lavy et al., 2015).

Isotopic $\delta^{15}N$ signatures and N:P ratios were determined with an isotope ratio mass spectrometer and a photometer, respectively, from dried coral tissue and extracted *Symbiodinium* cells previously separated by centrifugation and collected on filters. $\delta^{15}N$ signatures of dried material relative to atmospheric N were analyzed with an isotope ratio mass spectrometer (Lesser et al., 2007). Further, TN-to-TP ratios were measured photometrically following Hansen and Koroleff (2007).

2.5 | Microbial community composition and diazotroph abundance

For coral-associated bacterial community analysis, coral tissue was separated from the coral skeleton by airbrushing, and DNA from coral tissue was subsequently isolated with the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany) as per manufacturer's instructions. The relative abundance of tissue-associated diazotrophs was estimated based on relative gene copy numbers of the *nifH* gene in relation to 16S rRNA gene copy numbers. For this, qPCR amplifications of both genes were performed and the fold change of relative abundance of diazotrophs was calculated based on the $2^{(-\Delta\Delta Ct)}$ method.

Further, changes in the overall bacterial community composition in the coral tissue were determined using MiSeq 16S rRNA gene amplicon sequencing. Sequences were processed with MOTHUR v1.36.1 according to the MiSeq SOP (accession date: February 13 2017; Schloss et al., 2009). For a detailed description of the pipeline, please refer to the Supplementary Information. All sequence data are accessible under NCBI's BioProject ID PRJNA335276 (http://www.ncbi.nlm.nih.gov/bioproject/335276).

2.6 | Seawater nutrient measurements

Treatment water samples for nutrient analysis were collected at all sampling points. Treatment water was sampled in 30 and 50 ml triplicates for organic and inorganic nutrients, respectively, filtered (0.45 μm) and preserved with 100 μl of 35% phosphoric acid or frozen at −20°C, respectively. Analysis of DOC was performed with an Apollo 9000 Total Organic Carbon (TOC) Analyzer™ (Teledyne Instruments Tekmar, USA), and TN and TP concentrations were simultaneously measured according to standard method (SM) 4500-P J (Valderrama, 1981). Samples were analyzed by the Analytical Core

Lab (ACL) at KAUST, Saudi Arabia, and the Marine Chemistry Lab at the University of Washington, USA, respectively.

2.7 | Statistical analysis of physiological parameters and bacterial communities

All statistical analyses of physiological response parameters were conducted in R v3.2.2 (R Development Core Team, 2015). N_2 fixation, P_G, and R rates, as well as maximum quantum yield, were tested for individual and interactive effects of treatment and time by 2-factorial generalized estimation equations generalized linear models for repeated measures (GEEGLMs) in the R package GEEPACK (Højsgaard, Halekoh, & Yan, 2006). Similarly, *Symbiodinium* density, seawater nutrient concentrations, and *nifH* gene copy numbers were tested with 2-factorial generalized linear models (GLMs). Stable isotope composition and N:P ratios were analyzed in 3-factorial GLMs accounting for individual and interactive effects of treatment, time, and compartment. All models were based on a Gamma distribution with best fitting link function to account for skewing of data. To illustrate significant differences between manipulations, treatment effects of individual time points were compared using unpaired Welch's unequal variances *t*-test. Bacterial community composition was compared using analysis of molecular variance (AMOVA) as implemented in MOTHUR. All data are reported as mean ± *SE*; asterisks indicate statistically significant differences (*$p < .05$, **$p < .01$; for details, see Table S3).

3 | RESULTS

3.1 | Stimulated N_2 fixation, diazotroph abundance, and elemental changes

Within 7 days of manipulation, DOC additions caused a significant fourfold increase in holobiont gross N_2 fixation activity (assessed via acetylene reduction assay) compared to controls during both light and dark phase (Figure 1a and b; for all model statistics, see Table S3). Light N_2 fixation activity was higher and more variable compared to dark conditions in both treatments at all times. In contrast to N_2 fixation activity, the relative abundance of diazotrophs (relative number of *nifH* gene copies as quantified by qPCR) did not exhibit significant changes until day 28, but then experienced a 23-fold increase under high DOC compared to same-day ambient controls (Figure 1c).

Stimulated N_2 fixation activity concurred with a significant depletion of the isotopic $\delta^{15}N$ signature and a 40% increase in the N:P ratio over time for *Symbiodinium* under high DOC (Figure 2). In contrast, coral tissues maintained stable $\delta^{15}N$ signatures over time, but exhibited a doubling in the N:P ratio under stimulated N_2 fixation over the course of the experiment.

3.2 | Overall bacterial community

While diazotroph populations proliferated in the coral tissues, the overall bacterial community did not exhibit any significant compositional changes under high DOC over the course of the experiment (Fig. S1; Table S4). The overall community was dominated by Gammaproteobacteria (78%–85% of all sequences) for both treatments and across all time points. While an overall decrease in bacterial diversity was observed over time in both treatments (Table S5), no enrichment in potentially pathogenic bacterial families, such as the opportunistic Vibrionaceae (class Gammaproteobacteria) as previously reported from corals under DOC enrichment and coral bleaching and disease (Rosenberg & Falkovitz, 2004; Vega Thurber et al., 2009), was found (see Table S6 for abundances of operational taxonomic units (OTUs)).

3.3 | Coral bleaching

While corals in the control treatment maintained a healthy appearance (Figure 2a), corals in the DOC treatment experienced a pronounced paling over the course of the manipulation (Figure 2b).

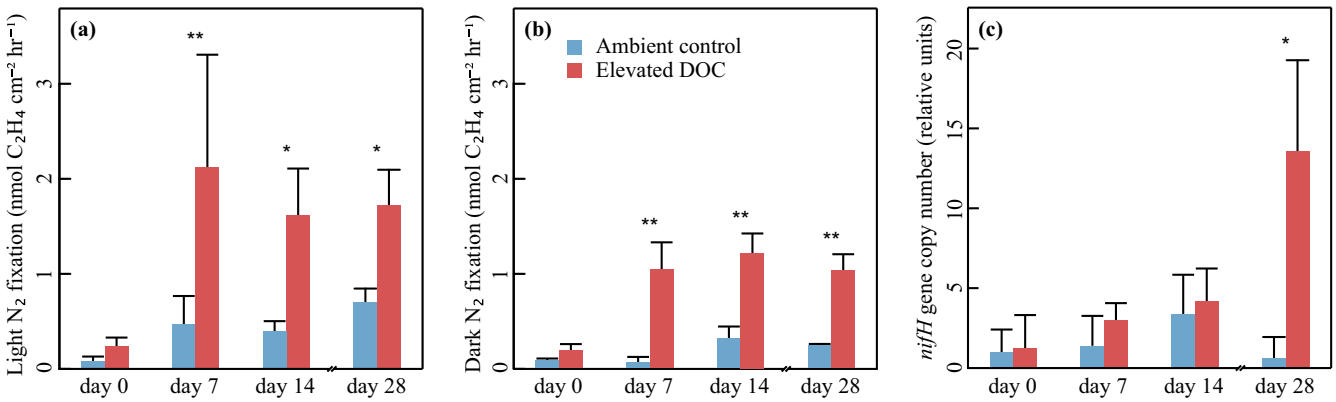

FIGURE 1 Dinitrogen (N_2) fixation activity and responses of coral-associated N_2-fixing bacteria to stimulation with labile dissolved organic carbon (DOC). (a and b) Light and dark coral-associated N_2 fixation rates expressed as ethylene (C_2H_4) evolution ($n = 6$ each). (c) Relative fold change in copy numbers of the *nifH* gene referenced to the 16S rRNA gene and in relation to day 0 control samples ($n = 3$ each). All data are presented as means ± *SE*. Asterisks indicate statistically significant differences (*$p < .05$, **$p < .01$). For full model statistics, see Table S3

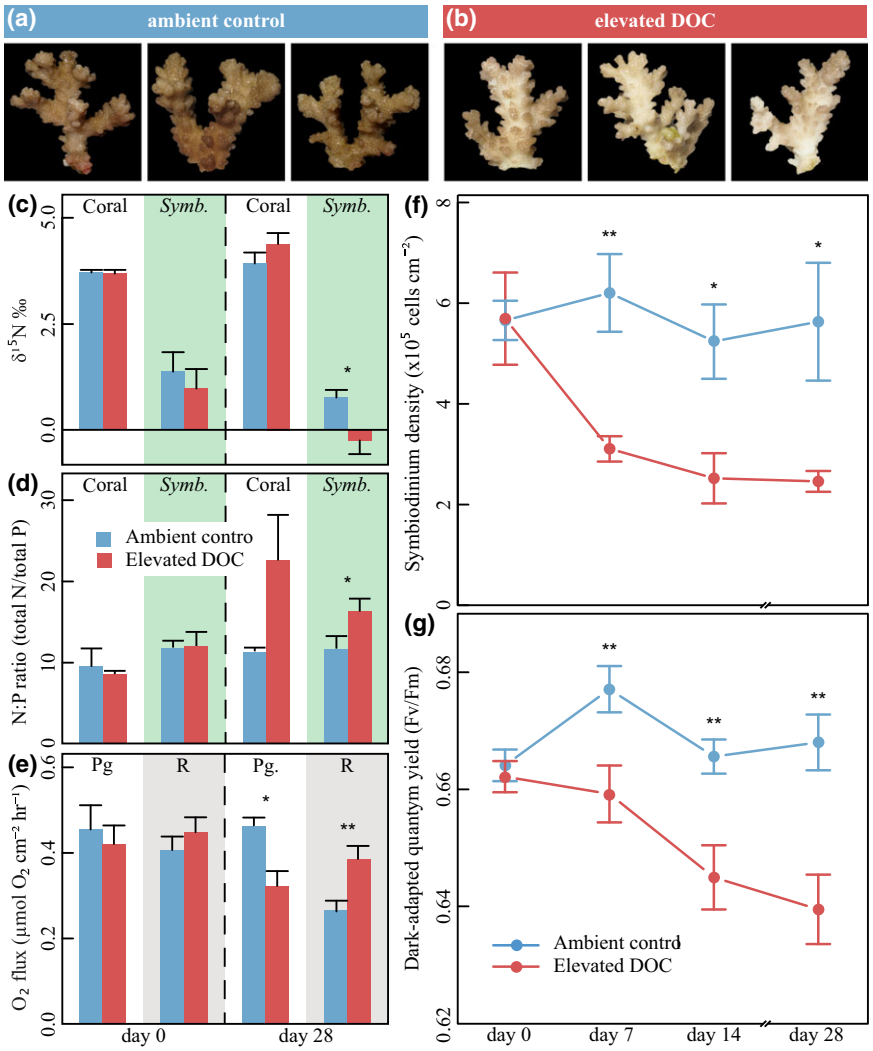

FIGURE 2 Physiological responses to labile dissolved organic carbon (DOC). Coral fragments subjected to (a) ambient control or (b) DOC enrichment. (c) Stable nitrogen ($\delta^{15}N$) signatures and (d) total nitrogen to total phosphorus ratio (N:P) of coral tissue and *Symbiodinium* (N = 3 each), (e) gross photosynthetic (P_G) and respiration (R) rates before and after 28 days of treatment (N = 3 each). (f) *Symbiodinium* densities *in hospite* (*n* = 3) and (g) fluorescent maximum quantum yield of photosystem II (F_v/F_m; *N* = 12) over the course of the experiment. All data are presented as means ± *SE*. Asterisks indicate statistically significant differences (*$p < .05$, **$p < .01$). For full model statistics, see Table S3

These visual symptoms were accompanied by a 60% loss of symbiotic algal cells within 28 days, as well as a small but highly significant reduction in the maximum quantum yield of photosystem II (PS II) of the algal symbionts (Figure 2f and g). These symptoms coincided with a 30% decline in holobiont gross photosynthesis, contrasted by a 40% increase in respiration rates (Figure 2e).

4 | DISCUSSION

4.1 | Coral bleaching in the absence of heat stress

The observed loss of algal symbionts coupled with changes in photosynthetic and nutrient cycling properties in DOC-stressed corals is strikingly similar to bleaching in thermally stressed corals. Therefore, the mechanisms involved in both bleaching phenotypes may share some important characteristics. Marked decreases of both, photosynthetic O_2 evolution and maximum quantum yield, are early responses of *Symbiodinium* to heat stress following the overwhelming of photoprotective mechanisms (Jones, Hoegh-Guldberg, Larkum, & Schreiber, 1998). The drop in maximum quantum yield in the present study was small (i.e., an order of magnitude lower compared to bleached corals), yet significant, and occurred in the absence of high temperature and light stress. While this small decrease may likely not be of ecological significance, it suggests the existence of mechanisms affecting the susceptibility of PSII to environmental stress. Among these mechanisms, an increase in the susceptibility to photodamage in corals due to P depletion (or starvation) under excess N conditions as proposed by Wiedenmann et al. (2012) would be plausible. Hence, the observed drop in photosynthetic efficiency may reflect early symptoms of disrupted N limitation in these corals.

Strong reductions in photosynthetic efficiency during heat-induced bleaching are well documented (Wiedenmann et al., 2012). Even though the photosynthetic efficiency experienced a significant reduction, it remained at an overall high level. Hence, this response is not comparable to heat stress responses and not indicative of photodamage and the associated accumulation of reactive oxygen species (ROS). As the upregulation of photosynthetic ROS production is a central mechanism of current bleaching theories, the apparent absence of oxidative stress raises the question about the exact trigger of bleaching in the present study (Weis, 2008). Similarly, Tolleter et al. (2013) reported coral bleaching in the dark during heat stress, that is, in the absence of excess photosynthetically derived ROS. Taken together, our results imply that excess photosynthetic ROS production is not necessarily required to initiate coral bleaching, and other sources of ROS production (e.g., mitochondria) or alternative causes of symbiont expulsion (e.g., retention of photosynthates) will have to be considered (Baird, Bhagooli, Ralph, & Takahashi, 2009).

4.2 | The role of the microbiome

As our observation of DOC-induced bleaching is in apparent contradiction with prevailing theories of bleaching (i.e., in the absence of photosynthetic ROS production), the mechanism of symbiotic breakdown in the current study deserves further elaboration. Previous studies linked the detrimental effects of DOC enrichment on corals to the opportunistic growth of heterotrophic (pathogenic) bacteria, virulence gene expression, and the formation of hypoxic layers on the coral surface (Kline, Kuntz, Breitbart, Knowlton, & Rohwer, 2006; Kuntz, Kline, Sandin, & Rohwer, 2005; Smith et al., 2006; Vega Thurber et al., 2009). Here, however, we show that the composition of the bacterial community of *P. verrucosa* remained stable despite elevated DOC conditions over 28 days. While this does not rule out possible changes in total bacterial abundance, it suggests strong regulatory forces within the holobiont inhibiting the opportunistic growth of potential pathogens. Instead, the microbiome was dominated by two bacterial OTUs of the genus *Endozoicomonas* at all times. These Gammaproteobacteria were repeatedly identified as highly prevalent and abundant associates of healthy corals, while reductions in their abundance may indicate unfavorable environmental conditions (Morrow et al., 2014; Roder et al., 2015). Notably, *Endozoicomonas* were recently suggested to have a major role in structuring coral microbiomes among other putative functions (Neave, Apprill, Ferrier-Pagès, & Voolstra, 2016; Neave, Michell, Apprill, & Voolstra, 2017; Neave, Rachmawati, Xun, Michell, Bourne, Aprill, & Voolstra, 2017).

In contrast to the overall stable bacterial community composition, the pronounced response of diazotrophs highlights that the DOC enrichment stimulated this functional group and potentially affected the activity of other holobiont-associated microbes. Stimulated diazotroph proliferation and N_2 fixation activity of similar magnitude have previously been reported for heat-stressed corals (Cardini, et al., 2016; Santos et al., 2014), thereby substantiating the proposition of a linkage between N_2 fixation and coral bleaching. Previous studies suggested that N_2 fixation in corals is energy-limited (Rädecker et al., 2014; Shashar et al., 1994). Consequently, labile DOC addition likely provided a readily available energy source for the metabolism and proliferation of coral-associated heterotrophic N_2 fixers (Olson, Ainsworth, Gates, & Takabayashi, 2009; Olson & Lesser, 2013). Additionally, hypoxic conditions due to increased bacterial respiration on the coral surface (Kline et al., 2006; Kuntz et al., 2005; Smith et al., 2006) may have promoted N_2 fixation, as the enzyme catalyzing the reaction, nitrogenase, is highly sensitive to O_2 availability (Compaoré & Stal, 2010). However, all corals showed highest N_2 fixation activity during active photosynthesis implying that coral-associated diazotrophs were capable of protecting the nitrogenase enzyme from O_2 evolution. Hence, energy rather than O_2 availability may be the dominant driver of diel N_2 fixation activity in the coral holobiont. Noteworthy, N_2 fixation activity in the DOC treatment increased before a relative proliferation of diazotrophs was observed in the tissue. This implies that diazotroph proliferation may have occurred elsewhere earlier in the experiment (e.g., in the mucus or coral skeleton). Further, this suggests that N_2 fixation activity in the holobiont may be limited by energy and environmental conditions rather than diazotroph abundance.

Importantly, due to the absence of potential pathogen propagation in the overall stable bacterial community, we can effectively rule out pathogenicity as suggested in previous studies (Kuntz et al., 2005; Smith et al., 2006). Consequently, the observation of increased N_2 fixation under these conditions may provide a mechanistic insight into the processes leading to symbiosis breakdown.

4.3 | The fate of microbially fixed nitrogen

Although the exact localization of diazotrophs within *P. verrucosa* remains yet to be determined, the stimulated N_2 fixation activity likely provided excess N to the coral holobiont. Indeed, the depletion in $\delta^{15}N$ in *Symbiodinium* suggests the direct utilization of N_2 fixation products at significant rates in the *Pocillopora verrucosa* holobiont, as reported previously for other corals (Lesser et al., 2007). While the underlying mechanism(s) of the transfer of N_2 fixation products to *Symbiodinium* remain(s) elusive, Benavides et al. (2016) recently showed that the direct transfer of fixed N and heterotrophic ingestion of diazotrophs provides a non-negligible and important N source for *Symbiodinium*. This uptake of additional N from N_2 fixation can explain the observed 40% increase in the N:P ratio in *Symbiodinium* cells in the current study, which are in general constant (Ferrier-Pagès, Godinot, D'Angelo, Wiedenmann, & Grover, 2016). Further, the shift in algal symbiont nutrient stoichiometry suggests that excess N uptake released *Symbiodinium* from their N-limited state, an important regulatory mechanism maintaining the coral–algae symbiosis (Falkowski et al., 1993).

As $\delta^{15}N$ signatures in coral tissue did not exhibit depletion, we can effectively rule out that increased N_2 fixation provided a significant source of N to the coral host within the experimental time frame. Still, coral tissue N:P ratios experienced an increase steeper than that of the algal symbionts. Although speculative at this point,

this may hint toward buffering mechanisms. Specifically, the coral host likely "sanctions" N supply to *Symbiodinium* by withholding N from its own metabolism, possibly to restore N limitation and prevent P starvation. These mechanisms may involve the storage of N derivatives in specialized host cells or organelles as previously suggested (Aranda et al., 2016; Pernice et al., 2012).

Taken together, our findings suggest that stimulated N_2 fixation altered the internal nutrient stoichiometry in the coral holobiont and disrupted the N-limited state of *Symbiodinium*. In this context, Godinot, Ferrier-Pagés, and Grover (2009) and Ezzat et al. (2016) previously reported shifts toward net release of dissolved inorganic N coupled with increased P and decreased N uptake in heat-stressed coral holobionts. This implies that shifts in internal nutrient stoichiometry may not be exclusive to DOC-induced bleaching, suggesting similar underlying processes may be involved during heat stress-induced (thermal) bleaching.

4.4 | A putative role of microbial N_2 fixation in coral bleaching

While the exact mechanism triggering symbiont expulsion requires further clarification, our findings do not contradict the prevailing bleaching theories, but rather extend our current understanding. Hence, we here propose a mechanistic concept integrating the observed detrimental role of stimulated N_2 fixation activity into the existing model(s) of (thermal) bleaching (Figure 3). This extended model posits that high temperatures (heat stress) or elevated DOC levels both stimulate nitrogenase activity and diazotroph proliferation, thereby increasing N_2 fixation activity (Cardini, Bednarz et al., 2016; Santos et al., 2014). The increased and preferential uptake of excess (microbially) fixed N releases the resident *Symbiodinium* population from N limitation, subsequently stimulating nutrient-balanced growth or even shifting algal symbionts to relative P depletion (P starvation). Although the mechanism of symbiont expulsion manifested as coral bleaching remains unknown at this point, here we demonstrate that the disruption of N limitation alone can rapidly result in the loss of algal symbionts. As the present study was not confounded by heat and light stress, the reported effects will likely be dramatically pronounced under these conditions.

Excess N supply from stimulated N_2 fixation could ultimately induce P starvation in *Symbiodinium*. Such shifts in the N:P ratio promote alterations in the symbiont's thylakoid membrane composition, increasing its susceptibility to photodamage. Simultaneously, the disruption of N limitation of *Symbiodinium* decouples the tight nutrient exchange relationship with the coral host (Dubinsky & Jokiel, 1994). As *Symbiodinium* will subsequently retain and channel most of their photosynthates into their own cell growth and repair, the coral host would be deprived of its main energy source. The resulting energy limitation of host CCMs would cause CO_2 limitation of photosynthetic dark reactions in *Symbiodinium*, thereby increasing their susceptibility to photodamage (Wooldridge, 2013). The consequential photosynthetic impairment and subsequent overproduction of ROS would cause further damage to the PSII and result in oxidative stress of both *Symbiodinium* and host cells (Weis, 2008).

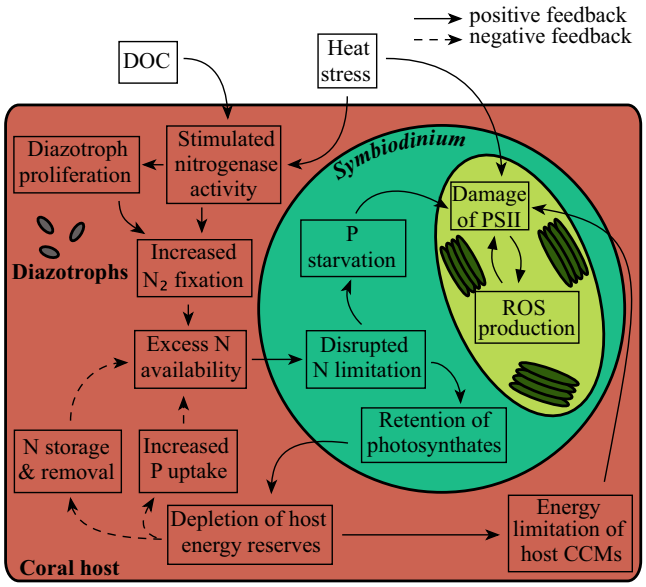

FIGURE 3 Conceptual model of the role of coral-associated dinitrogen (N_2) fixation in coral bleaching. High seawater temperatures or dissolved organic carbon (DOC) levels stimulate N_2 fixation activity in the holobiont. Excess fixed nitrogen (N) is rapidly taken up by *Symbiodinium*, inducing nutrient-balanced growth or even phosphorus (P) starvation. This P starvation would alter the composition of algal photosynthetic membranes, causing the photosystem II (PSII) of the photosynthetic apparatus to malfunction. Under heat stress, damage to PSII would promote the increased production of reactive oxygen species (ROS) in *Symbiodinium*. Simultaneously, the disruption of N limitation would increase the retention of fixed carbon by algal symbionts, forcing the coral host to deplete its own energy reserves. This could increase the susceptibility to photodamage due to a failure of host carbon concentration mechanisms (CCMs) causing CO_2 limitation of photosynthetic dark reactions. To restore control over the symbiosis, we hypothesize the coral host would attempt to increase its P uptake and/or to retain N from *Symbiodinium*.

Based on the strong increase in N:P ratios in the coral tissue in spite of the increase in $\delta^{15}N$, we hypothesize that the coral host simultaneously attempts to restore a stable nutrient exchange relationship by altering the nutrient supply to *Symbiodinium*. This could be achieved either by removal, assimilation, or storage of N derivatives in host cells or organelles, or by the upregulation of other microbial N cycling pathways (nitrification, denitrification) coupled with increased P uptake and translocation to *Symbiodinium* (Ezzat et al., 2016; Pernice et al., 2012; Rädecker et al., 2015).

Noteworthy, the present study was conducted in the absence of additional light or heat stress. Hence, the consequences of altered nutrient cycling would have likely resulted in a more pronounced stress response under these conditions. Ultimately, the threshold at which coral bleaching occurs likely depends on whether the intensity and duration of environmental stress exceed

the energetic capability of the coral host to maintain the N limitation of *Symbiodinium*.

4.5 | Ecological relevance of elevated DOC levels on coral reefs

Coral reefs can be regionally exposed to periodically changing levels of TOC/DOC and may range from low (~30 to 70 μM; Haas et al., 2016; on average 130 μM for ambient Red Sea water in the present study) to periodically high levels (as observed for some parts of the Caribbean and the Florida Keys; >1,000 μM; Kline et al., 2006; up to 1,600 μM; Boyer, Fourqurean, & Jones, 1997). The DOC enrichment in the present study achieved a more than tenfold increase (868–1,609 μM) relative to the untreated ambient control (117–154 μM) and therefore constitutes an ecologically relevant enrichment level at an order of magnitude increased in comparison with levels reported for coral reefs. It is worthwhile to note that biological replicates were supplied from a common reservoir of DOC enriched water, in order to exclude confounding effects from differences in DOC enrichment or degradation. This was necessary as labile DOC is rapidly consumed in an aquaria setup (Haas, Al-Zibdah, & Wild, 2009). A potential carry-over effect between corals and water coming from any of the aquaria was minimized via filtration, the use of protein skimmers, and a high renewal rate of seawater in the tanks. While we cannot positively exclude that coral microbiomes were affected by surrounding colonies (something also possible in the reef environment; see Roder, Bayer, Aranda, Kruse, & Voolstra, 2015), possible effects are assumingly minor in relation to the treatment effect. This is supported by the notion that microbial community compositions were maintained throughout the course of the experiment on the level of (1) replicate coral colonies, (2) between the control and treatment, and (3) that the seawater N and P content showed no differences between control and treatment over time. Nonetheless, the DOC enrichment caused a rapid significant shift in the N:P ratios of the two main eukaryotic departments of the *P. verrucosa* holobiont: the host and the algal symbiont. These changes were likely facilitated by the oligotrophic conditions of the Red Sea water used in this experiment. In a naturally less oligotrophic system, such as the Caribbean, higher DOC levels would likely be necessary to evoke equivalent responses (Kline et al., 2006). Nevertheless, DOC additions in the same order of magnitude as employed in the current experiment induced coral bleaching and mortality in corals from Panama and the Northern Gulf of Aqaba (Haas et al., 2009; Kline et al., 2006). Apart from these environmental factors, the effects of DOC enrichment on coral holobionts also depend on its quality and composition. While a large fraction of DOC in the Caribbean is refractory and of terrestrial origin (Lirman & Fong, 2007; Nebbioso & Piccolo, 2013), the current and previous manipulative studies employed mostly labile DOC sources. As labile DOC is readily available for microbial utilization, its overall effects on the coral holobiont are different from those of refractory DOC. Labile DOC is introduced onto coral reefs from various sources. Municipal sewage and algal exudates, for instance, contain significant proportions of labile DOC of similar saccharide composition as in the present study (Huang et al., 2010; Nelson et al., 2013). Indeed, macroalgae exudates differentially enrich and stimulate bacterial cell growth, favoring the prevalence of opportunistic and potentially pathogenic bacteria, induce coral mortality, and cause shifts toward less efficient copiotrophic reef bacterial communities (Haas et al., 2016; Nelson et al., 2013; Smith et al., 2006).

4.6 | Coral reef resilience in a changing ocean

Coral-associated N_2 fixation is increasingly being recognized as beneficial for coral health (Rädecker et al., 2015) and fundamental for sustaining primary productivity under (seasonally) changing environmental conditions (Cardini et al., 2015; Cardini, van Hoytema et al., 2016; Rädecker et al., 2015). On the other hand, we show here that diazotroph activity can destabilize the coral–algae symbiosis and thus may pose a threat to overall holobiont functioning. While the current study used DOC enrichment to induce bleaching, our findings may be applicable to thermal bleaching as similar responses of the diazotroph community appear to be in place. Thermal bleaching has long been recognized as one of the most severe threats to modern coral reefs (Hughes et al., 2003). Our findings imply that the ubiquitous presence of diazotrophs in most coral holobionts may pose a threat to corals in a warming ocean. However, similar as in thermal bleaching, changes in the coral-associated N_2 fixation activity and its impact on holobiont functioning will be largely dependent on the environmental (i.e., holobiont) context (Grottoli, Rodrigues, & Palardy, 2006).

Reshef, Koren, Loya, Zilber-Rosenberg, and Rosenberg (2006) suggested that a restructuring of the coral microbiome may facilitate the rapid adaptation of coral holobionts to changing environmental conditions (see also Ziegler, Seneca, Yum, Palumbi, & Voolstra, 2017). Therefore, a reduction in diazotroph abundance or activity could potentially enhance the thermal tolerance of corals in a warming ocean. In the long term, however, the coral's ability to evolve may be hampered by its complex mutualistic relationship with *Symbiodinium*, rendering scenarios likely in which rapid global climate change outpaces the coral's capacity for adaptation (Pandolfi, Connolly, Marshall, & Cohen, 2011).

There may be no rapid solution to reduce the effects of global climate change in the near future. All the more important becomes mitigation, for example, by reducing local anthropogenic stressors, in future conservation efforts. Here, we show that DOC enrichment can rapidly stimulate the N_2 fixation pathway in the coral *P. verrucosa*. Based on this, we argue that the stimulation of N_2 fixation via sewage and wastewater may be a possible mechanism rendering reef-building corals more susceptible to the effects of global environmental change, particularly heat stress. At the same time, the role of N_2 fixing bacteria for holobiont functioning largely depends on environmental N availability. While helping to sustain productivity during low N availability, stimulated N_2 fixation together with environmental N enrichment may destabilize holobiont functioning. To provide a better understanding of the mechanism

proposed in this study, follow-up work will have to validate and extend the experiments conducted, including other coral species that cover different coral functional groups as well as a range of environmental stressors causing bleaching. Further, the mechanism proposed here may not be applicable to coral bleaching responses induced by factors other than DOC or heat stress (e.g., cold stress-induced bleaching).

The detrimental effects of labile DOC on reef-building corals, however, remain non-negligible (Kuntz et al., 2005; Kline et al., 2006; Smith et al., 2006; Haas et al., 2009; this study). Thus, a priority in local management efforts should be the reduction of DOC input and loading on coral reefs. Sources of DOC enrichment on coral reefs include sewage, wastewater, and excessive algal abundance (Smith et al., 2006; Wear & Thurber, 2015). Consequently, to diminish microbially driven reef degradation processes, management measures would benefit best from combined efforts (Haas et al., 2016; Zaneveld et al., 2016). Specifically, improved wastewater facilities to effectively retain inorganic and organic nutrients coupled with the restoration of herbivorous fish stocks to control for harmful algal growth would likely increase the resilience of corals to ocean warming (Vega Thurber et al., 2012, 2014; Wear & Thurber, 2015).

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AUTHOR CONTRIBUTIONS

CP, NR, AC, CRV, and CW designed research. AG, CRV, CW contributed reagents and tools. CP, NR, AC performed research, and CP and NR analyzed data. CP, NR, CRV, and CW interpreted data. CP, NR, AC, CRV, and CW wrote the manuscript. All authors read and approved the final manuscript.

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None declared.

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SUPPORTING INFORMATION

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