Coral holobiont functioning under global environmental change

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Here briefly, learning, one with nature. Memories swim ever gently.

- Peter Sale

‘So, like it or not, microbiology is going to be in the center of evolutionary study in the future—and vice versa.’

‘The time has come to replace the purely reductionist ‘eyes-down’ molecular perspective with a new and genuinely holistic, eyes-up, view of the living world, one whose primary focus is on evolution, emergence, and biology’s innate complexity.’

- Carl R. Woese
SUMMARY

The tropical scleractinian coral holobiont is comprised by the coral animal, dinoflagellate algae of the genus *Symbiodinium*, and a multitude of other microbes, specifically bacteria, archaea, viruses, and protists. These holobionts are the unit of ecological selection, and remarkably adapted to thrive under oligotrophic (nutrient-poor) conditions. The foundation for this adaptation is provided by the coral-algae symbiosis, a mutualistic nutrient exchange relationship between the coral and *Symbiodinium*, allowing for the high primary productivity and growth rates of tropical coral holobionts. The coral-algae symbiosis is maintained via nitrogen limitation by the host, and new nitrogen from heterotrophic feeding, dissolved nutrient uptake, or coral-associated nitrogen fixation activity is retained and taken up within the holobiont. Excess nitrogen (EN) availability however can rapidly tip this delicate balance and disrupt the coral-algae symbiosis. The investigation of functional dependencies of individual holobiont members to nitrogen cycling as well as responses of individual nitrogen cycling pathways to the effects of global environmental change is therefore critical to understanding overall coral holobiont functioning. (Summarized and reviewed in Chapter 1).

Chapter 2 addresses the effects of internal (i.e., within the holobiont) nitrogen (ammonia) availability due to stimulated nitrogen fixation activity in a 28 day *ex situ* experiment on the scleractinian *Pocillopora verrucosa* holobiont. Using a manipulative elevated DOC enrichment approach, and taking the responses of the holobiont, the coral host, *Symbiodinium*, and the prokaryotic nitrogen fixing community into account, this chapter provides qualitative evidence for dramatic cascading effects induced by stimulated holobiont-associated nitrogen fixation activity. This chapter demonstrates that the uptake of microbially fixed EN can indeed release *Symbiodinium* from nitrogen limitation, thereby rapidly inducing a phenotype of coral bleaching in the absence of heat and light stress, and, ultimately, host mortality. The findings further suggest the existence of potential buffering mechanisms by the animal, probably employed to restore nitrogen limitation under EN conditions. Finally, these findings are linked to coral stress responses under heat stress in a conceptual model proposing a mechanistic key role of stimulated nitrogen fixation activity in thermal bleaching. Chapter 3 however is based on the *P. verrucosa* holobiont as a model system, but does not resolve differential association with nitrogen fixation in a coral comparative taxonomic framework.

Consequently, Chapter 3 explores the physiological and functional basis of nitrogen fixation in four widely distributed coral species from two Indo-Pacific coral families, the Pocilloporidae and Fungiidae. This was achieved by measuring the associated nitrogen fixation rates, abundance of nitrogen fixing microbes, and the active transcription (gene expression) of the *nifH* gene, a common biomarker for nitrogen fixing prokaryotes. Findings show marked
differences on the family level, which interestingly align with the overall stress susceptibility of the investigated coral families. While more empirical data are needed at this point, Chapter 4 gives rise to the idea that coral-associated nitrogen fixation may be a driver of stress susceptibility and therefore resilience in tropical scleractinian corals. This chapter postulates the existence of two co-existing, but likely non-exclusive ecological strategies based on fixed nitrogen acquisition: high versus low nitrogen fixation-dependence. Corals of the second group could potentially exhibit a higher capacity to exploit nitrogen sources other than microbial nitrogen fixation, e.g. heterotrophic feeding, more efficiently.

Finally, Chapter 4 addresses the responses of the main members of the *P. verrucosa* holobiont to external EN availability (i.e., eutrophication via DON provision) over 14 days of exposure. While a strong functional dependency on nitrogen availability was apparent in the *Symbiodinium* population, as reflected in dramatic and rapid numerical and compositional responses, the overall bacterial community composition remained stable even during the onset of mortality. This finding suggests the presence of strong host selective forcing to maintain an invariant holobiont prototype. The prevalence of *P. verrucosa* in the Red Sea suggests a high ecological ubiquity of this prototype characterized by a broad ecological niche, yet low competitiveness. Emerging from these findings and the extant literature, the highly selected *P. verrucosa* holobiont appears to further have a rather low acclimatization capacity, which may impede its ability to cope with rapid global environmental change in the long term.

This thesis highlights the importance of functional dependencies on nitrogen cycling, particularly the nitrogen fixation pathway, in coral holobiont functioning, and the importance of employing a large set of response parameters covering critical functions of the main holobiont members. Based on such an interdisciplinary approach, this thesis found that the responses of coral-associated nitrogen fixing microbes and changes in their associated activity may destabilize holobiont integrity under the effects of global environmental change. Thereby, nitrogen fixation could pose a previously overlooked threat to coral holobionts. Particularly in holobionts exerting strong host regulative forcing on their microbial communities, specific holobiont compositions may therefore be unable to adapt to future ocean scenarios, and rapidly succumb to the effects of global environmental change. Collectively, the work presented in this thesis emphasizes the necessity to reduce the amount of (in)organic nutrient input, particularly organic carbon and (in)organic nitrogen, into sensitive coastal waters in order to mitigate the effects of global climate change, and to preserve coral reefs in the future.
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LIST OF ABBREVIATIONS

16S rRNA a section of the prokaryotic ribosomal DNA found in all Bacteria and Archaea.

δ^{15}N Expression of the nitrogen stable isotope ratio in delta notation calculated relative to atmospheric nitrogen as: \( \delta^{15}N = \left( \frac{R_{\text{sample}}}{R_{\text{Reference}}} - 1 \right) \times 1000 \), where Reference = 15N/14N and Reference = 0.00368‰

\( \%_\circ \) Parts per mille, the units in which \( \delta \) values are expressed

ARA Acetylene Reduction Assay

C\(_2\)H\(_2\) Acetylene

C\(_2\)H\(_4\) Ethylene

C:N ratio carbon to nitrogen ratio

Chl \( \textit{a} \) Chlorophyll \( \textit{a} \)

DNA Desoxyribonucleic acid

DOC Dissolved organic carbon

DON Dissolved organic nitrogen

DOM Dissolved organic matter

EN Excess Nitrogen

F\(_{\text{v}}\)/F\(_{\text{m}}\) Maximum (fluorescence) quantum yield

ggelm Generalized Estimating Equations; a Generalized Linear Model suitable for repeated measurement designs

glm Generalized Linear Models

ITS2 Internal Transcribed Spacer 2. A genetic region commonly applied to resolve the phylogenetic relationships within the Eukaryota.

N\(_2\) Dinitrogen

N:P ratio Nitrogen : Phosphorus ratio


NH\(_4^+\) Ammonium

\( \textit{nifH} \) a subunit of the \( \textit{nif} \) gene cluster, which encodes for the two identical subunits of the denitrogenase reductase enzyme in microbial nitrogen fixation. Found in Bacteria and Archaea

NO\(_3^-\) Nitrate

OM Organic matter
P Phosphorous
PAM Pulse Amplitude Modulation
PAR Photosynthetically active radiation (wavelength 400 – 700 nm)
PCR polymerase chain reaction
P_g Gross photosynthesis
P_N Net photosynthesis
R Respiration
RNA Ribonucleic acid
ROS Reactive Oxygen Species
TOC Total organic carbon
qPCR quantitative PCR
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CHAPTER 1

General Introduction: Coral holobionts in a changing world
Coral Holobionts in a Changing World

The Coral Reef Ecosystem

Tropical coral reefs occupy less than 0.1% of the ocean surface (1). Yet, they are home to more than 25% of marine species (2) and support the livelihoods of more than 500 million people with their ecosystem services (3). One particularly notable feature of coral reefs, however, is their exceptionally high productivity in commonly oligotrophic (nutrient-poor) tropical waters (4–6). Coral reef primary production is several orders of magnitude higher than that of the surrounding water column (4, 7), and rivals that of rainforests, the most productive terrestrial ecosystems (8). This seemingly contradictive observation has been coined ‘The Darwin Paradox’ in reference to its first observer (9, 10). The explanation for this paradox likely lies in the particularly efficient uptake and recycling of nutrients within the reef (11–13).

Despite their historical ecological success, coral reefs have experienced an unprecedented global decline over the past decades (14). About 75% of the world’s coral reefs are threatened (15), and predictions suggest that more than 60% of coral reefs may be lost as early as 2030 (16, 17). This decline has been attributed to a series of anthropogenic global (i.e. ocean warming and acidification) and local (e.g. nutrient enrichment and overfishing) stressors, which can act in addition to natural disturbances, be chronic, and may increase the frequency of natural disturbances (18). Commonly, these stressors do not occur isolated on coral reefs, but may co-occur and interact with each other, thereby exacerbating individual stressor effects (16, 19–21). For a better understanding of how coral reefs respond to the effects of individual or combined stressors, however, it is important to further our understanding of their key ecosystem engineers, scleractinian corals.

Coral Holobionts as Ecosystem Engineers

Scleractinian corals are holobionts constituting an ecological unit comprised by the coral animal (or host), dinoflagellate algal symbionts of the genus *Symbiodinium*, and a multitude of other microbes, including bacteria, archaea, protists, and viruses (22, 23). This complex inter-kingdom association can help extend the physiological capabilities of each of its members alone (23), and determine the ecological success of the entire holobiont (24). Owing to the coral-*Symbiodinium* mutualism, for instance, coral holobionts are able to precipitate their internal calcareous skeletons, literally the foundation of tropical coral reefs are built, at high rates. Therefore, scleractinian corals are considered key reef engineers, as they shape entire ecosystems of impressive spatial scales via the accretion of the coral reef substrate. These three-dimensional structures affect the physical, chemical, and biological environment, and provide
structural complexity and hence shelter for a multitude of pro- and eukaryotic organisms (25, 26).

There is more to corals than meets the eye, though. Due to their intimate association with their microbial (e.g., bacterial and other) community, corals have a critical importance in coral reef biogeochemical processes (11, 27–32). Indeed, scleractinian corals widely contribute to nutrient cycling on coral reefs via the generation and transformation of inorganic and organic materials (11, 12, 26, 27, 33). Specifically, corals release considerable amounts of dissolved and particulate organic matter into the water column, which may function as energy carrier and particle trap in the oligotrophic reef waters (11, 34–36). Finally, while their contribution to the benthic fixed nitrogen budget on the reef scale is minor (37), corals contribute heavily to carbon cycling on coral reefs, which appears to be strongly coupled with nitrogen cycling (38). Therefore, degraded reefs are associated with altered nutrient cycling (26, 30, 39, 40). Understanding the susceptibility of corals to the effects of anthropogenically-driven global environmental change and how these responses will shape the coral reefs of the future is therefore critical to the development of meaningful management purposes.

The Coral-Algae Symbiosis: Maintenance and Breakdown

The coral-algae symbiosis is the foundation upon which the million-year long ecological success of tropical coral reefs is built (41). This intimate nutrient exchange symbiosis is characterized by a tight recycling and conservation of nutrients within the holobiont. Specifically, the photoautotrophic *Symbiodinium* translocate the majority of their net fixed inorganic carbon (photosynthates) to the coral host (42, 43). In return, the algal symbionts are provided with inorganic metabolic by-products from the coral, and acquire dissolved inorganic nitrogen from the surrounding water column (41, 44), mostly in the form of ammonium (NH$_4^+$) and nitrate (NO$_3^-$) (45). The photosynthates translocated to the host by *Symbiodinium* can fully cover or even exceed the coral’s metabolic requirements (46). While up to 50 % of the net fixed carbon is subsequently released as coral mucus (11, 27), the better part remains within the holobiont. As these photosynthates consist mostly of glycerol, glucose, and possibly lipids, they are characterized by a high carbon to nitrogen ratio (37, 47).

The growth rate of *Symbiodinium* is potentially much higher than that of coral cells. In culture, doubling time averages 2-5 days, while in hospite, this process is considerably slower (≥ 10 days, and may exceed 70 days). As for the symbiosis to persist in a steady state, the growth rates of host and symbionts must keep pace with each other (48). Consequently, the existence of the following internal regulation mechanisms controlling for the proliferation of *Symbiodinium* within the host cells has been suggested: (i) host release factors (HRFs), a group of host-derived compounds, likely amino acids, triggering the translocation of carbon-rich photosynthates to
the coral (49–51); (ii) degradation or digestion of *Symbiodinium* cells in hospite (52); (iii) symbiont expulsion (53); and (iv) inhibition of symbiont cell growth and division by resource limitation. Among these mechanisms, particularly the limitation of nitrogen availability as a regulatory means of *Symbiodinium* growth rates in hospite has received much attention (54).

For corals, the intimate partnership with *Symbiodinium* is associated with distinct costs. The symbiosis is prone to disruption when subjected to certain environmental stressors (55), notably thermal and light stress (56), or poor water quality (57). Symbiotic disruption encompasses coral bleaching or disease, two major drivers of global reef degradation (55, 58). Thermal bleaching is attributed to high temperature anomalies in combination with high light intensity (56, 59, 60), and typically lies at only 1°C above the maximum average summer temperature (55, 61). During bleaching, the density of *Symbiodinium* or their photosynthetic pigment concentrations are greatly reduced, for instance via the expulsion of (often functionally competent and structurally intact) *Symbiodinium* cells (62, 63). Consequently, the underlying white calcium carbonate skeleton of the coral animal becomes apparent (56, 64). Bleached corals are commonly physiologically impaired, exhibiting reduced growth, respiration, and photosynthetic rates, increased disease susceptibility, and mortality (65–67). While mass bleaching can have devastating effects on the reef scale (16), surviving corals can acquire new algal symbionts once the causative environmental stressor abates before mortality occurs (68).

The environmental drivers of coral bleaching are well documented. Still, our understanding of the underlying suborganismal and cellular processes remains incomplete. The Oxidative Theory of Bleaching proposed the involvement of reactive oxygen species (ROS) in the algal cell via damage to the photosynthetic apparatus (69, 70). Subsequent ROS leakage into the host cells rapidly overwhelms the coral’s antioxidant capacity under stress (56). The bleaching cascade is finally initiated by the stimulation of innate immune-like pathways via the combined upregulation of reactive nitrogen species in the host gastrodermal cell (71, 72), ultimately leading to symbiotic breakdown (56, 60). Recent findings however suggest coral bleaching can occur even without the involvement of light-induced oxidative stress at elevated temperatures alone, in absolute darkness (73), and distinct mismatches in the upregulation of antioxidant defences of host and *Symbiodinium* preceding bleaching (74) are apparent. This suggests alternative forces may be at work in bleached coral holobionts.

The *Symbiodinium* population is regulated via nitrogen limitation in hospite (46, 75). Increased availability of inorganic nitrogen can therefore constitute a detriment to coral health, even though scientific consensus remains ambiguous. While a smaller pool of studies suggests that nutrient enrichment is not detrimental for reef functioning (76), recent findings strongly suggest that not only the concentration of inorganic nutrients in coral reef waters, but also changes in the nutrient stoichiometry and/or species may severely affect coral health. Increases
in the seawater nitrogen to phosphorous (N:P) ratio can induce nutrient-imbalanced growth in *Symbiodinium*, resulting in the destabilization of its thylakoid membranes, thereby increasing the holobiont’s susceptibility to bleaching (77). Nitrogen species on the other hand may affect the rates of carbon acquisition, fixation, and translocation by *Symbiodinium* (78). Finally, elevated nutrient concentrations can increase bacterial opportunism and mortality in parrotfish feeding marks at elevated temperatures, thereby turning normal trophic interactions disastrous for corals (21).

**Importance of Bacteria and other Microbes in the Coral Holobiont**

Understanding the complex host-microbe interactions underlying holobiont functioning has been dubbed a new frontier in zoology (79). Scleractinian coral holobionts are associated with highly diverse microbial communities, consisting upwards of several thousand distinct bacterial phylotypes (80). Therefore, coral holobionts range among the most diverse meta-organisms known to date (80). Coral-associated bacterial communities are highly complex and uneven, and differ distinctly in their composition from the surrounding water column or other reef substrates (81–84). Within the holobiont, the different functional departments - surface mucus layer, coral tissue, gastro-vascular cavity, and coral skeleton - are occupied by distinct bacterial communities (85–87), which can exhibit intra-departmental spatial heterogeneity (88). Coral microbiomes commonly are species-specific (22, 84) and conserved across oceans (89, 90), but may be dramatically altered in bleached or diseased corals (microbial dysbiosis; (91), which can exhibit distinct shifts towards a more opportunistic and potentially pathogenic community composition (82, 92–96). Consequently, coral microbiomes were suggested to be of critical importance for holobiont functioning and health (23, 97–99).

In this context, the Coral Probiotic Hypothesis postulates the rapid restructuring of the coral-associated microbial community within days to weeks to facilitate acclimatization of corals to environmental change (100). While the coral-associated community potentially covers and provides a multitude of functions and services to coral holobiont functioning (23, 101, 102), little is known about the significance of individual bacterial functional groups for coral health. Mucus-associated bacteria have previously been suggested to act as a first barrier of defence against pathogen entry (22, 103) by potentially occupying entry niches and/or through the production of secondary metabolites, preventing the dominance of single and potentially pathogenic bacteria (104). Functional genes related to key biogeochemical cycles, more specifically carbon, nitrogen, sulphur and phosphorus metabolism were reported in association with corals (97, 98, 102, 105, 106). It has therefore been suggested that coral-associated bacteria are critical to nutrient cycling and retention in the holobiont (29, 47, 102, 107–109). As nitrogen is a key limiting element in the coral-algal symbiosis, the role of nitrogen cycling
bacteria has gained scientific attention in recent years (47, 110). While there is evidence for nearly complete nitrogen cycles in the cold-water scleractinian holobiont *Lophelia pertusa* (111) and octocoral *Anthothela* holobionts (112), there is very little information available on its individual pathways in corals, even for the fairly well-investigated nitrogen fixation pathway.

**Coral Holobiont-Associated Microbial Nitrogen Fixation**

Dinitrogen (N\(_2\)) is the most abundant gas in the atmosphere of Planet Earth. As it is extremely unreactive (environmentally inert), it is only accessible to a small but diverse prokaryotic functional group, the diazotrophs, which encompass a multitude of Bacteria and Archaea (113). These prokaryotes are capable of the chemical reduction of N\(_2\) into the equivalent of ammonia, a nitrogen species which can be readily incorporated into biological molecules (114). This process is commonly referred to as microbial nitrogen fixation or diazotrophy. Microbial nitrogen fixation constitutes the most energy-consuming means of ammonium assimilation, requiring 16 mol of ATP for the reduction of 1 mol of N\(_2\) (115). This reaction is catalysed by the nitrogenase enzyme complex (114). Nitrogenase is highly sensitive to oxygen, which can affect its protein structure and inhibit its synthesis (116). This oxygen sensitivity in combination with molecular work indicate that all extant nitrogenases are derived from a single common ancestor, which has likely emerged prior to the Great Oxygenation Event (117).

Nitrogen fixation is widely associated with benthic coral reef organisms and substrata (10, 29, 38, 108, 118–121), including scleractinian corals (29, 109, 110, 122, 123), and is, within the tropical coral holobiont, the most widely studied nitrogen transformation pathway. As the photosynthates translocated by *Symbiodinium* have a high C:N ratio, corals require “new” external nitrogen, (46, 124). Therefore, the provision of fixed nitrogen via diazotrophy is considered highly critical for holobiont functioning (47, 110). Indeed, coral holobionts ubiquitously harbour a diverse community of nitrogen fixing microorganisms including cyanobacteria (47) and, mostly, heterotrophic bacteria (123, 125–128). Within the coral holobiont, the resident *Symbiodinium* population constitutes a sink for microbi ally-fixed nitrogen (110, 127, 129). Nitrogen fixation may therefore play an important role in sustaining coral productivity during periods of low external nitrogen availability (29). Additionally, coral-associated diazotroph communities are coral species-specific, highly conserved over space and time (123, 125), and can be vertically transmitted, i.e. from parental colonies to larvae (126). While the magnitude of transfer of fixed nitrogen from diazotrophs into other compartments of the coral holobiont (e.g. *Symbiodinium*) remains yet to be quantified, recent studies show that bacterial symbionts readily supply the holobiont with nitrogen (110, 129, 130), and can meet up to 11% of the nitrogen requirements of *Symbiodinium* at times of low environmental
ammonium availability on a high latitude coral reef (29). Consequently, nitrogen fixation is widely believed to be beneficial for holobiont functioning under unperturbed conditions (29, 30).

**The Effects of Environmental Change on Coral-Associated Nitrogen Fixation**

Coral-associated microbial nitrogen fixation responds rapidly to changing environmental conditions, such as seasonality in temperature or nutrient availability (29, 38, 120). Consequently, anthropogenically-driven environmental change (i.e., the effects of global climate change and local stressors, such as pollution and nutrient enrichment) will likely affect the magnitude of nitrogen fixation activity and possibly other nitrogen transformation pathways in corals (40, 131) (Figure 1). Indeed, Rädecker et al. (109) reported a rapid and significant decrease (53 %) in diazotrophy in the pocilloporid coral *Seriatopora histrix* exposed to ocean acidification treatments (pCO2 1080 μatm) over the course of several days only. In contrast, elevated seawater temperatures were reported to rapidly stimulate the nitrogen fixation pathway up to 300 % in the corals *Acropora hemprichi* and *Stylophora pistillata* (30).

Figure 1. Hypothesized nitrogen cycling in the coral holobiont representing the major nitrogen transformations under unperturbed conditions and under anthropogenically-driven environmental change (40). (A) nutrient-depleted (oligotrophic; ‘pristine’), (B) nutrient-enriched (eutrophic), and (C) heat stressed (elevated temperature) conditions. Bold white arrows indicate the anticipated relative amplification of individual nitrogen transformations compared to the oligotrophic scenario. Dashed arrows indicate the relative diminution of pathways compared to the oligotrophic scenario. It is hypothesized that changes in the nitrogen fixation pathway (coupled with changes in the other nitrogen transformation pathways) may aid in the population control of *Symbiodinium* under oligotrophic and eutrophic conditions, but may result in elevated nitrogen availability under heat stress. Abbreviation: org., organic. From Rädecker et al. (40).
The effects of other stressors on nitrogen fixation activity in corals however remain virtually unknown. One under investigated, but critical stressor potentially affecting nitrogen fixation in corals is dissolved organic carbon (DOC). While an early report described the restoration of inhibited coral nitrogen fixation via glucose addition (132), there is no information on the effects of elevated levels of DOC on coral-associated diazotrophs and their implications for the coral holobiont. DOC is part of the dissolved organic matter (DOM) pool, and can severely and detrimentally affect the performance and functioning of the holobiont (136). Critical in this context are the highly bioavailable or *labile* compounds of DOM, which are metabolized by the heterotrophic microbial community within minutes to days (137). Allochthonous OM is introduced into sensitive coral reef waters via the release of untreated or poorly treated sewage or municipal wastewaters (136, 138–140). Autochthonous sources on coral reefs include scleractinian and soft coral mucus and macroalgae exudates (11, 27, 28, 141–144). Both allochthonous and autochthonous dissolved organic carbon (DOC) can rapidly cause symptoms similar to thermal stress responses in corals, specifically the disruption of the coral-algae symbiosis, i.e., bleaching (134, 145), tissue lesions, and increased disease prevalence and mortality (133, 146). These deleterious effects are commonly attributed to increased bacterial growth and respiration, and, consequentially, the formation of oxygen-depleted (hypoxic) layers on the coral surface (133, 134, 145). High DOC exposure can also shift coral microbiomes towards more opportunistic and potentially pathogenic communities (dysbiosis), and increase virulence gene expression (147). In this context, it remains yet to be determined how microbial nitrogen fixation is affected by chronic exposure to high DOC, and how these changes will affect overall holobiont functioning.

*Linking Nitrogen Fixation with Coral Holobiont Breakdown and Reef Degradation*

Both elevated temperature and dissolved organic carbon (DOC) availability can result in coral bleaching and mortality (65, 133, 134, 145, 150, 151). Coincidentally, both stressors also stimulate coral-associated bacterial growth and respiration, including nitrogen fixation activity (30, 96, 122). Cardini et al. (30) suggested that this increase in nitrogen fixation could potentially ameliorate the effects of heat stress and bleaching in the coral holobiont, similar to heterotrophic feeding (152, 153). In contrast, Rädecker et al. (40) proposed it could disrupt nitrogen limitation in the holobiont, and thereby rapidly derail the coral-algae symbiosis and cause reef degradation (Figures 2, 3).

In this scenario, the stimulation of holobiont-associated nitrogen fixation and associated excess nitrogen availability for *Symbiodinium* is predicted to result in dramatic cascading effects on the coral-algae symbiosis, ultimately resulting in an overall holobiont breakdown (40).
(Figure 2). Briefly, the increased uptake of excess nitrogen fixation products by the algal symbionts would rapidly release them from nitrogen limitation, thereby inducing nutrient-imbalanced growth. The resulting increase in the *Symbiodinium* N:P ratio would ultimately lower the threshold at which coral bleaching occurs (39).

The downstream effects of stimulated nitrogen fixation in corals may not only act on the holobiont (or colony) level, but will likely have dramatic consequences at the ecosystem level (40). At the reef scale, the breakdown of the coral–algae symbiosis and subsequent holobiont mortality likely trigger the DDAM (*DOC, disease, algae, microbes*) positive feedback loop (133, 134, 145, 146, 154, 155) of coral reef degradation (Figures 2, 3) (40). Epilithic turf and macroalgal communities rapidly colonizing the bare skeletons of recently killed corals both exhibit high nitrogen fixation rates (37, 118) and release significant amounts of exudates rich in labile DOC (134, 142, 156). These exudates yet again stimulate microbial activity (133, 134, 145), including nitrogen fixation in nearby corals, thereby promoting large-scale ecosystem degradation (Figures, 2, 3) (40). It is therefore critical to elucidate the functional role of nitrogen fixation in the (de)stabilization of holobiont functioning under environmental stress.
Figure 2. The hypothesized role of nitrogen fixation in coral holobiont breakdown and ecosystem-scale reef degradation (40). (A) Elevated DOC concentrations and heat stress increase the overall microbial activity in corals, including nitrogen fixation. (B) Excess microbially fixed nitrogen induces nutrient-imbalanced growth of the endosymbiotic alga *Symbiodinium*. (C), resulting in a lowered threshold at which coral bleaching occurs. (D) Upon coral mortality, the bare coral skeleton will be overgrown by a succession of turf and macroalgae, which release DOC-rich exudates (DDAM feedback loop (133, 134, 145, 146, 154, 155). Thereby, algae stimulate microbial activity including nitrogen fixation in nearby corals, promoting reef degradation at an ever-increasing scale (154, 155). From Rädecker et al. (40).
Figure 3. Reef degradation by coral-algae interactions at different spatial scales (40). Progression of coral mortality on the (A) coral colony level and (B) community to ecosystem level. The release of DOC-rich algae exudates is a mechanism stimulating coral-associated microbial activity (133, 134, 145) that likely also increases nitrogen fixation. It was recently postulated that stimulated nitrogen fixation could be a driver of rapid disruption of the coral–algae symbiosis. From Rädecker et al. (40)
Knowledge Gaps

An increasing number of studies describe coral-associated bacterial communities, but only few reports tackle the functional aspects of these assemblages (102). The enigmatic diazotrophs for instance are of critical importance for coral primary productivity (29, 40), but our understanding of their responses to selected anthropogenic stressors as well as their role in holobiont functioning and breakdown under environmental change remains poor.

No previous efforts have undertaken combined assessments of the molecular basis of nitrogen fixation in corals. Specifically, there are no expression-based studies linking active nifH transcription with actual nitrogen fixation rates, and no previous efforts in a comparative coral taxonomic framework. Therefore, there is no information on potential emerging patterns of nitrogen fixation among different groups of coral, and whether nitrogen fixation is omnipresent in all corals (Question 1; Chapter 3).

Elevated seawater temperatures can increase coral-associated nitrogen fixation rates. However, there is still debate whether this has ameliorative (30) or detrimental effects (40) on holobiont functioning during bleaching, and what mechanisms are underlying the bleaching response (Question 2; Chapter 2). Further, it needs to be determined how holobiont breakdown under bleaching differentiates from other stressors, for instance EN (Question 3; Chapters 2, 4).

Despite the increasing number of studies targeting coral-associated bacterial communities, their complex responses to global environmental change remain poorly understood. In this context, it remains unclear whether changes in the bacterial community are always acclimative or even adaptive (The Coral Probiotic Hypothesis (100) or pathogenic (147, 150, among others), or if other ‘strategies’ of bacterial community responses exist in corals, ultimately determining the success of a stress response to environmental change (Questions 4, 5; Chapter 4).

Holistic experimental designs taking the responses of the holobiont, the coral animal, Symbiodinium, and the coral-associated bacterial community into account will be required to tackle these gaps. The preliminary answers provided in this thesis will contribute towards a better understanding of coral and reef stress responses to global environmental change, and ultimately aims to aid in the development of meaningful management actions.
Specific Objectives and Questions

This thesis aims to address scleractinian coral stress responses to environmental change in a comprehensive holobiont framework. The overall goal is to further our understanding of the functional dependencies on nitrogen cycling of the complex inter-kingdom relationships within the coral holobiont. In order to achieve this, the following questions were addressed:

1) How does high DOC availability affect coral-associated nitrogen fixation? What are the ecological implications?
2) What is the role of nitrogen fixation in coral holobiont breakdown (i.e., bleaching)? What are the potential implications of changes in nitrogen fixation activity on the ecosystem level?
3) How is holobiont breakdown manifested under high DOC and EN provision? Is there a functional dependency of the individual members of the holobiont on nitrogen cycling?
4) Is there evidence for host forcing in the *P. verrucosa* holobiont on the *Symbiodinium*, bacterial, and diazotroph communities under high DOC and EN availability? What are the implications of diazotroph dependency and a highly selected holobiont composition for the stress susceptibility and acclimatization potential in *P. verrucosa* in a changing world?
Approach

The experiments for this work were carried out under controlled conditions in closed aquaria systems at the wet lab facilities of the Core Lab for Marine Operations and Research (CMOR) of the King Abdullah University of Science and Technology (KAUST), Kingdom of Saudi Arabia. The model organisms, multiple colonies or individuals of the common scleractinian coral families Pocilloporidae (Pocillopora verrucosa, Stylophora pistillata) and Fungiidae (Pleuractis granulosa, Ctenactis echinata), were collected on the exposed sites of the midshore reef al Fahal (22°18.22.6N 38°57.494E) and the inshore reef Fsar (22°13.974N, 39°01.760E), respectively, located in the Central Red Sea off the Saudi Arabian coast.

In order to gain a comprehensive picture of holobiont responses to changes in the (in)organic nutrient environment (elevated DOC; excess nitrogen availability), response parameters were selected to cover critical functions of all departments of the Pocillopora verrucosa holobiont. The selected response parameters were as follows:

- **Holobiont**: dark respiration rates (R).
- **Coral animal**: calcification rates; tissue stable isotope (δ\(^{15}\)N) signatures; total nitrogen to phosphorus (N:P) ratio.
- **Symbiodinium**: gross photosynthetic rates (G\(_{\text{r}}\)); maximum quantum yield (F\(_{\text{v}}\)/F\(_{\text{m}}\)); cell density; relative chlorophyll \(a\) (chl \(a\)) content; stable isotope (δ\(^{15}\)N) signatures; total N:P ratio; composition of the Symbiodinium community (next-generation sequencing of the ribosomal ITS2 region).
- **Coral tissue-associated bacterial community**: nitrogen fixation rates (measured indirectly via ethylene evolution in the acetylene reduction assay); bacterial community composition (next-generation sequencing of the 16S rRNA gene on the Illumina miseq platform); abundance of the diazotroph community (quantitative PCR of the 16S rRNA, ITS2 and \(nifH\) genes).

Further, the physiological and molecular basis of nitrogen fixation was compared in the two coral families Pocilloporidae and Fungiidae. The following response parameters were assessed in a control and high DOC treatment:

- Nitrogen fixation rates (measured indirectly via ethylene evolution in the acetylene reduction assay),
- diazotroph abundance (relative copy numbers of the \(nifH\) gene, qPCR application),
- active transcription of the \(nifH\) gene (gene expression rates; qPCR application).

The tools and methods applied to tackle these response parameters are described in detail in the respective chapters.
THESIS OUTLINE

This thesis consists of a general introduction (Chapter 1), three chapters presenting the core research of the PhD (Chapters 2-4), and a general discussion (Chapter 5). Chapters 2-4 are intended for publication as independent research articles in international peer-reviewed journals. Additional co-authored publications that are related to the thesis, but not included in it are listed below and the abstracts are presented at the end of the thesis.

Chapter 2:

Stimulated nitrogen fixation causes coral bleaching in the absence of heat and light stress.

Pogoreutz†, C., Rädecker†, N., Cárdenas, A., Gärdes, A., Voolstra*, C.R., Wild*, C.

† shared contribution
* shared contribution

This chapter approaches the conceptual model presented in Chapter 1 in a 4 week ex situ experimental manipulation. Using a standardized source of DOC, the aim of this study was to demonstrate the intimate link between coral-associated nitrogen cycling and holobiont functioning, and thereby to elucidate the critical role of the microbial nitrogen fixation pathway in coral symbiotic breakdown. Specifically, a large set of physiological and molecular parameters covering vital functions of the main members of the coral holobiont, specifically the coral animal, Symbiodinium, and the coral tissue-associated bacterial community as well as several measures on the holobiont level were selected to gain a holistic picture of their interactions. Based on our findings, we provide a new conceptual model of microbe-mediated coral bleaching that can even occur in the absence of heat and light stress.

This study was conceived by C. Pogoreutz, N. Rädecker, A. Cárdenas, C.R. Voolstra, and C. Wild. C. Pogoreutz developed the research questions and hypotheses and designed the experiments with input from N. Rädecker, A. Cárdenas, C.R. Voolstra, and C. Wild. Sampling and experimental work was conducted by C. Pogoreutz with support from N. Rädecker and A. Cárdenas. C. Pogoreutz analyzed the samples with support from N. Rädecker and A. Cárdenas. C. Pogoreutz analyzed the data and wrote the manuscript with input from all co-authors. This chapter is currently prepared for submission to Nature Ecology and Evolution.
Chapter 3:

Do differences in bacterial nitrogen fixation align with stress susceptibility in reef-building corals?


† shared contribution  
* shared contribution

This chapter provides a first account of the physiological and molecular basis of nitrogen fixation in tropical scleractinian corals in a comparative taxonomic framework. This study combines the traditional acetylene reduction assay approach for the indirect determination of nitrogen fixation activity with quantitative PCR (qPCR) approaches of the *nifH* gene, a common biomarker for nitrogen fixing prokaryotes. This was achieved by linking coral-associated nitrogen fixation rates with the abundance of nitrogen fixing bacteria (assessed via the qPCR determination of relative gene copy numbers) and active transcription of the *nifH* gene (qPCR approach to assess relative gene expression rates). Thereby, this study shows clear differential physiological and molecular patterns evidently conserved in two families of tropical scleractinian corals. We further propose a link between the patterns in nitrogen fixation activity with stress susceptibility in these families and propose that nitrogen fixation activity may render corals more susceptible to global environmental change.

This study was conceived by C. Pogoreutz, N. Rädecker, C. Wild, and C.R. Voolstra. C. Pogoreutz developed the research questions and hypotheses and designed the experiments with input from N. Rädecker, C.R. Voolstra, and C. Wild. Sampling, experimental work and data analysis was conducted by C. Pogoreutz with support from N. Rädecker. C. Pogoreutz analyzed the data and wrote the manuscript with input from all co-authors. This chapter is in preparation for submission to *Environmental Microbiology*. 
Chapter 4:

*Invariance of bacterial community composition under excess nitrogen indicates limited ability to holobiont adjustment in the coral *Pocillopora verrucosa*

**Pogoreutz, C., Rädecker, N., Cárdenas, A., Gärdes, A., Wild*, C., Voolstra*, C.R.**

*shared contribution*

This chapter aims to experimentally investigate the functional nitrogen dependency of the main members of the coral holobiont by taking measurements of the holobiont, the coral animal, *Symbiodinium*, and the tissue-associated bacterial community into account. We hypothesize that high nitrogen provision rapidly derails the coral-algae symbiosis, which is accompanied by considerable compositional responses of the *Symbiodinium* and bacterial community. Based on our findings, we propose that the physiologically plastic *P. verrucosa* holobiont exhibits an exceptional host selectivity to maintain its potentially symbiotic core microbiome. While this evolutionary strategy may contribute to the high success of *P. verrucosa* along the entire Red Sea Basin, especially on moderately disturbed reefs, it may hinder rapid acclimatization or adaptation responses under rapid environmental change.

This study was conceived by C. Pogoreutz, N. Rädecker, C. Wild, and C.R. Voolstra. C. Pogoreutz developed the research questions and hypotheses and designed the experiments with input from N. Rädecker, C.R. Voolstra, and C. Wild. C. Pogoreutz conducted the experimental work with support from N. Rädecker. C. Pogoreutz analyzed the samples with support from N. Rädecker and A. Cárdenas. C. Pogoreutz analyzed the data and wrote the manuscript with input from all co-authors. This chapter is in preparation for submission to *mBio*. 
RELATED PUBLICATIONS


REFERENCES


90. Chu ND, Vollmer S (2016) This article is protected by copyright. All rights reserved. *Environ Microbiol Rep* 2–43.


Chapter 1


CHAPTER 2

Stimulated nitrogen fixation causes coral bleaching in the absence of heat and light stress

ABSTRACT

As sea surface temperatures increase, the disruption of the coral-algae symbiosis (coral bleaching) has become an unprecedented threat to tropical coral reefs worldwide. In a stable state, this symbiosis is maintained by constant nitrogen limitation of the algal symbionts. Conversely, excess nitrogen availability can destabilize this symbiosis and lower the thermal bleaching threshold of corals. Still, the role of microbial nitrogen cycling processes and their effects on the coral-algal symbiosis during bleaching is unknown to date. Here we induced a coral bleaching phenotype in the absence of heat and light stress by stimulating nitrogen fixation activity in holobiont-associated microbes (Bacteria and Archaea) with labile dissolved organic carbon (neutral monosaccharides). $\delta^{15}$N signatures confirmed the rapid uptake of nitrogen fixation products by algal symbionts, releasing them from nitrogen limitation as reflected by increased nitrogen to phosphorus ratios. Concomitantly, symbiotic breakdown occurred as reflected by loss of algae, reduced photosynthetic performance, and ultimately coral bleaching. Based on these findings, we propose a conceptual model of coral bleaching integrating microbial nitrogen fixation into current prevailing theories. Our study highlights the critical role of this ubiquitous prokaryotic functional group, commonly considered beneficial under pristine conditions, in coral reef degradation processes in future ocean scenarios.
SIGNIFICANCE STATEMENT

Elevated sea temperatures and high irradiance can cause the breakdown of the symbiosis between reef corals and their intracellular photosynthetic algae known as coral bleaching. Despite its critical relevance under global environmental change, our understanding of the underlying causes remains incomplete. Here we identify a mechanism linking the increased growth and activity of coral-associated nitrogen-fixing microbes (Bacteria and Archaea) with coral bleaching even in the absence of heat and light stress. Our findings emphasize the critical role of coral-associated nitrogen-fixing microbes as potential drivers of reef degradation, thereby providing novel management opportunities for the mitigation of climate change effects on coral reefs.
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 (1). In this mutualistic association, the coral host provides inorganic nutrients in exchange for photosynthetically fixed carbon (photosynthates) and amino acids from the algal symbiont (1). The disruption of this delicate symbiosis by heat and light stress (2) or poor water quality (3, 4), commonly referred to as coral bleaching, may ultimately result in mortality of the coral host. Mass coral bleaching events have resulted in unprecedented degradation of coral reefs over the past decades (5). Further, the frequency and severity of these events appears to increase as global climate change progresses (6).

Even though several decades have passed since the initial observation of coral bleaching, our understanding of the mechanistic processes remains incomplete. Among the proposed mechanisms, particularly the Oxidative Theory of Bleaching (OTB) has found considerable resonance. The OTB suggests that the bleaching cascade is initiated by oxidative stress developing in the algal symbionts due to increased light and temperature conditions (7–11). Yet, there is emerging evidence for additional mechanisms intimately linking bleaching with environmental nitrogen availability (12–15). As a constant nitrogen limitation of *Symbiodinium* is required to regulate algal cell division rates and promote the translocation of photosynthates to the coral, nitrogen enrichment may threaten the persistence of this symbiosis (16, 17). Specifically, increased seawater nutrient availability can reduce the translocation rates of photosynthates by the algae (2, 18, 19). Accordingly, Wooldridge (18) proposed that this retention of organic carbon would cause a subsequent energy limitation of coral host carbon concentrating mechanisms (CCMs). The resulting carbon dioxide (CO₂) limitation of photosynthetic “dark reactions” would render *Symbiodinium* more susceptible to photodamage and thus ultimately to coral bleaching. Indeed, this ‘selfish symbiont’ mechanism was recently supported by Ezzat et al. (19), who reported the increased utilization and reduced translocation of carbon by *Symbiodinium in hospite* under high or imbalanced nutrient scenarios. Additionally,
Wiedenmann et al. (14) showed that such nutrient imbalance can lower the bleaching threshold in corals. Specifically, excess nitrogen availability shifts *Symbiodinium* to a phosphorus-starved state, promoting the substitution of phospholipids with sulpholipids in the thylakoid membranes (20). As the membrane lipid composition of the thylakoid can be a driver of bleaching sensitivity in *Symbiodinium* (21), increased nitrogen availability likely results in the improper assemblage and functioning of their photosystem II (PSII), ultimately rendering the coral holobiont more susceptible to bleaching (14).

Despite the increasing recognition of the importance of nitrogen availability, an understanding of internal nitrogen cycling processes in corals during thermal bleaching is lacking to date. This information is critical, as nitrogen cycling microbes are ubiquitous members of the coral microbiome (22, 23). In particular, diazotrophs, more specifically members of the two prokaryotic domains Bacteria and Archaea capable of reducing dinitrogen (N₂) into biologically available ammonium, provide an important nitrogen source for *Symbiodinium* (24–27). Indeed, nitrogen fixation can help sustain holobiont productivity when nutrients are scarce (28). Given its functional importance, it is not surprising that active nitrogen fixation has been reported in association with the majority of investigated coral species (28–32). Increased temperatures however stimulate the enzymatic activity of nitrogenase (33) and promote the proliferation (34) and activity (35) of coral-associated diazotrophs. The combined picture emerging from these findings has recently led Rädecker et al. (23) to propose that excess nitrogen availability from increased holobiont-associated nitrogen fixation activity during heat stress may be a major driver of thermal bleaching in corals.

Here, we aimed to provide a mechanistic understanding of the role of nitrogen fixing Bacteria and Archaea during coral bleaching. For this purpose, we manipulatively stimulated nitrogen fixation activity in corals in the absence of heat or light stress. This approach allowed us to identify the effects of increased nitrogen fixation activity on the coral-algal symbiosis, whilst eliminating the confounding effects of temperature and irradiance. To achieve this, we supplied doses of labile dissolved organic carbon (DOC), more specifically neutral monosaccharides (for
details see Methods and Supplementary Methods), to stimulate coral-associated nitrogen fixation, as reported previously (31). While DOC can cause symbiotic breakdown and mortality in corals at elevated concentrations (36, 37), a mechanistic explanation of the underlying processes is missing to date. We thus conducted a controlled 28-day laboratory study on the symbiotic coral *Pocillopora verrucosa*, a common model organism for coral physiology studies (37–41). Coral fragments were acclimated in 6 replicated closed natural reef water tank systems in the absence of heat and light stress. Subsequently, half of the tanks were treated with daily dosings of DOC, while the other half remained non-manipulated controls. The addition of DOC produced a >10 fold enrichment in the treated aquaria and resulted in a coral bleaching phenotype over the course of the 28-day manipulation, while corals in the untreated seawater remained visibly healthy (for full details on seawater parameters and model statistics, see Supplementary Tables S1 and S2). We characterized the response of a suite of physiological parameters covering critical functions of the coral holobiont (42) and three of its main members – the coral host, algal symbionts, and the prokaryotic microbial community, specifically the bacterial community as well as the nitrogen fixing microbes (Bacteria and Archaea). While our findings do not contradict the main hypothesis of bleaching, they provide a relevant comprehensive extension. Specifically, here we show the putatively critical role of coral-associated nitrogen fixing microbes in reef degradation processes with implications for reef management in the mitigation of global climate change effects.

**RESULTS AND DISCUSSION**

**Stimulated nitrogen fixation, diazotroph abundance, and overall bacterial community**

Within 7 days of manipulation, DOC additions caused a significant 4-fold increase in the holobiont nitrogen fixation activity compared to controls, both in the light and dark phase (as evident from acetylene reduction assays; Fig. 1 *A* and *B*; for full model statistics, see Supplementary Table S3). Light nitrogen fixation activity was consistently higher and more variable compared to dark conditions. Of note, this stimulation of nitrogen fixation activity is of
similar magnitude as reported from heat stressed and bleached Red Sea coral holobionts (3-fold increases; 35). Since biological nitrogen fixation is a highly energy-consuming process, requiring 16 mol of ATP for the reduction of one mol of N\textsubscript{2} into ammonium (43), previous studies suggested that nitrogen fixation in corals may be energy-limited (31, 32). Consequently, labile DOC addition likely provides a readily available additional energy source for the metabolism and proliferation (36, 37) of coral-associated heterotrophic nitrogen fixing bacteria (25, 44–46).

Indeed, we measured a 23-fold increase in the relative number of \textit{nifH} gene copies under high DOC (Fig. 1 C) indicating a proliferation of nitrogen fixing Bacteria and/or Archaea in the coral tissue towards the end of the experiment. Similarly, an increase in the coral-associated diazotroph abundance of the same order of magnitude (13- to 27-fold) was previously observed in heat stressed corals (34), substantiating the proposition of a linkage between nitrogen fixation and coral bleaching. Importantly, nitrogen fixation rates increased independently of diazotroph abundance (as measured by qPCR) as evident from the temporal mismatch between the increase in activity and abundance of diazotrophs. Thus, nitrogen fixation activity in corals appears to be first and foremost limited by energy availability. Of note, DOC can also stimulate heterotrophic microbial activity other than nitrogen fixation, for instance accelerated bacterial cell growth and respiration (36, 37). As a consequence, hypoxic (oxygen-depleted) layers may form on the coral surface (37). Nitrogenases, the key enzymes catalyzing the reduction of nitrogen to ammonium, are highly sensitive to oxygen (47). Therefore, hypoxic conditions may favor increased nitrogenase activity, and contribute to stimulated nitrogen fixation. While it was previously suggested that DOC-induced hypoxia (36), microbial activity (37), or virulence gene expression (12, 48) cause coral bleaching and mortality, this does not contradict the evidently detrimental effects of increased nitrogen fixation on the holobiont. Rather, the observation of increased nitrogen fixation under these conditions may provide a mechanistic understanding of these processes.
Figure 1. Dinitrogen (N₂) fixation activity and responses of coral-associated N₂-fixing bacteria to stimulation with labile dissolved organic carbon (DOC). (A and B) Light and dark coral-associated N₂ fixation rates expressed as ethylene (C₂H₄) evolution (n = 6 each). (C) Relative fold change in copy numbers of the \textit{nifH} gene normalized to 16S rRNA reference gene and in relation to day 0 control samples (n = 3 each). All data are presented as means ± SE. Asterisks indicate statistically significant differences (*) \( P < 0.05 \), (**) \( P < 0.01 \). For full model statistics, see Supplementary Table S3.

Despite the dramatic increase in the coral tissue-associated diazotroph abundance after 28 days, the overall bacterial community did not exhibit significant compositional changes in response to the DOC treatment over 28 days (Supplementary Figure S1; for full amova details, see Supplementary Table S4/5). Concomitantly, no enrichment in members of Vibrionaceae (class Gammaproteobacteria) or other putatively pathogenic bacterial families previously reported from corals under DOC enrichment (12) and coral bleaching and disease (49, 50) was observed (Supplementary Table S6). This finding contrasts with previous studies reporting significant decreases in the dominant bacterial classes Alpha- and Gammaproteobacteria in the coral \textit{Porites compressa} exposed to glucose additions in the same order of magnitude, albeit at smaller time scales (7). Indeed, in the current experiment, the coral tissue-associated bacterial communities in both treatments and time points were dominated by two genotypes of Endozoicomonaceae. These Gammaproteobacteria were previously identified as highly prevalent associates of scleractinian corals, but appear to be particularly dominant in the Pocilloporidae (51–54). As Endozoicomonaceae appear to negatively correlate with thermal bleaching (52), dominate the bacterial communities of Pocilloporidae throughout their entire global range, and are highly host specific in the genus \textit{Pocillopora} (54), they have been proposed...
to be obligate microbial symbionts (52, 54). These findings corroborate our view that not shifts towards a more pathogenic bacterial community composition, but rather changes in the activity and abundance of diazotrophs associated with *P. verrucosa* are drivers of holobiont responses to DOC in the current study.

**Elemental changes**

Stimulated nitrogen fixation activity concurred with a depletion of the stable nitrogen (δ¹⁵N) signature and an increase in the total nitrogen to phosphorus (N:P) ratio over time for *Symbiodinium* under high DOC (Fig. 2C and D). Since nitrogen fixation favors the lighter δ¹⁴N isotope via fractionation processes, studies assessing natural δ¹⁵N signatures have used its relative depletion to identify the transfer of fixed nitrogen from diazotrophs to *Symbiodinium* in corals (26). Whilst the underlying mechanism remains elusive, Benavides *et al.* (27) recently showed that the direct transfer of fixed nitrogen and heterotrophic feeding on diazotrophs provides a non-negligible and important nitrogen source for *Symbiodinium*. Hence, the depleted δ¹⁵N signatures in this study not only confirm the excess supply of fixed nitrogen derived from nitrogen fixation, but also the direct utilization of these products by the algal symbionts. As *Symbiodinium* N:P ratios are in general quite constant (55), this uptake of additional nitrogen can explain the observed 40% increase in the N:P ratio in *Symbiodinium* cells (Fig. 2D). This further suggests a rapid release of *Symbiodinium* from their nitrogen limited state, and the subsequent derailment of the coral-algae symbiosis.

In contrast, while coral tissue exhibited no significant differences in δ¹⁵N under stimulated nitrogen fixation, its N:P ratio doubled. As δ¹⁵N signatures were not depleted, we can effectively rule out the increased uptake and utilization of nitrogen from nitrogen fixation by coral host cells. Still, coral tissue N:P ratios experienced an increase steeper than that of algal symbionts. Although speculative at this point, this may hint towards buffering mechanisms by the coral host to remove excess nitrogen from *Symbiodinium* to restore nitrogen limitation and prevent detrimental phosphorus starvation. One possible buffering mechanism may involve the
storage of nitrogen derivatives in specialized host cells or organelles as suggested recently (56).

In this context, changes in the nutrient uptake stoichiometry may further help the coral host to buffer internal shifts in the N:P ratio. This view is supported by Godinot et al. (57) reporting a net release of dissolved inorganic nitrogen coupled with increased phosphorus uptake in the coral *Stylophora pistillata* under heat stress. Furthermore, whilst nitrogen fixation activity rapidly responded to DOC enrichment, diazotroph abundance remained constant at first and showed a significant increase after 28 days (Fig. 1 C). This mismatch suggests that diazotroph proliferation in the coral tissue was inhibited initially. Accordingly, this raises questions regarding the location of these microbes within the holobiont, as well as the role of host regulation in maintaining their population size. Overall, our findings suggest that nitrogen limitation of *Symbiodinium* could not be maintained over the course of the experiment, although increases in the host N:P ratio may have attenuated effects of altered nutrient availability for *Symbiodinium* and may hint towards potential buffering mechanisms.

**Coral bleaching in the absence of heat stress**

While corals in the control treatment remained in a healthy state (Fig. 2 A), corals in the DOC treatment experienced paling over the course of the manipulation (Fig. 2 B). These symptoms were accompanied by a 50 % loss of symbiotic algae cells within 28 days, along with a 30 % decline in gross photosynthesis (Fig. 2 E-F). These symptoms are strikingly similar to bleaching in thermally stressed corals, suggesting that analogous mechanisms may have been involved. Marked decreases of both, photosynthetic oxygen (*O₂*) evolution and the maximum quantum yield, are early responses of *Symbiodinium* to heat stress following the inundation of photoprotective mechanisms (2). The drop in maximum quantum yield in the present study was small, yet highly significant, and occurred in the absence of high temperature and light stress. While this minute decrease is likely of no ecological relevance for the coral holobiont, it suggests the existence of mechanisms affecting the susceptibility of PSII to environmental stress. In this context, Wiedenmann *et al.* (14) reported an increased susceptibility to photodamage in corals.
under relative P depletion (or starvation). Hence, the observed drop in photosynthetic efficiency may reflect initial symptoms of disrupted nitrogen limitation and potential phosphorus starvation in these corals.

Even though the photosynthetic efficiency experienced a significant drop, it remained at a high level not indicative of photodamage in the photosystem II (PSII) and the associated accumulation of reactive oxygen species (ROS). As the upregulation of photosynthetic ROS production is a central mechanism of prevailing bleaching theories, this raises the question regarding the triggering of bleaching in the present study (7, 14, 18). Similarly, Tolleter et al. (58) reported coral bleaching in the dark during heat stress, i.e. in the absence of photosynthetically derived ROS production. Taken together, these results imply that photosynthetic ROS is not necessarily required to initiate the bleaching cascade in corals. Alternatively, other sources of ROS production (e.g., mitochondria) or alternative causes of symbiont expulsion (e.g., retention of photosynthates) have to be considered.
Figure 2. Physiological responses to labile dissolved organic carbon (DOC). Coral fragments subjected to (A) control and (B) DOC enrichment. (C) Stable nitrogen (δ15N) signatures and (D) total nitrogen to total phosphorus ratio (N:P) of coral tissue and *Symbiodinium* (N = 3 each), (E) gross photosynthetic (Pg) 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 (Fv/Fm; N = 12) over the course of the experiment. All data are presented as means ± SE. Asterisks indicate statistically significant differences (*P < 0.05, **P < 0.01). For full model statistics, see Supplementary Table S3.

The putative role of microbial N₂ fixation in coral bleaching

While the exact mechanism triggering symbiont expulsion requires further clarification, our findings are not necessarily in contradiction with prevailing bleaching theories. Rather, we here propose a mechanistic concept integrating the observed detrimental role of stimulated nitrogen fixation activity into the existing models of (thermal) bleaching (Fig. 3). This extended
model assumes that high temperatures (heat stress) or elevated DOC levels both stimulate nitrogenase activity and diazotroph proliferation, thereby increasing nitrogen fixation activity (28, 33, 34). The increased and preferential uptake of excess fixed nitrogen rapidly releases the resident *Symbiodinium* population from nitrogen limitation (23), subsequently stimulating either nutrient-balanced growth, or even shifting algal symbionts towards relative phosphorus depletion (P starvation) (14). Although the mechanism of symbiont expulsion remains speculative at this point, here we demonstrate that this disruption of nitrogen limitation alone may 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 exacerbated under such conditions.

The enhanced N supply could ultimately induce phosphorus starvation in *Symbiodinium*. This would promote alterations in the symbiont’s thylakoid membrane composition, thereby increasing their susceptibility to photodamage (14). Simultaneously, the disruption of nitrogen limitation of *Symbiodinium* would decouple the tight nutrient exchange relationship with the coral animal (17). As *Symbiodinium* would subsequently retain and channel most of its photosynthate into cell growth and repair rather than translocate them, the host would be deprived of its main energy source. The resulting energy limitation of host CCMs would cause CO₂ limitation of photosynthetic dark reactions in *Symbiodidinium*, thereby increasing its susceptibility to photodamage (18). The consequential photosynthetic impairment and subsequent overproduction of ROS (7) would cause further damage to PSII and result in oxidative stress of both *Symbiodinium* and host cells.

Based on the strong increases in the coral tissue N:P ratios despite an increase in δ¹⁵N, 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 and storage of nitrogen derivatives in host cells or organelles (56), or by the upregulation of other nitrogen cycling pathways (nitrification, denitrification; 23) coupled with increased phosphorus uptake and translocation to *Symbiodinium* (57), as the uptake assimilation of both nutrients appears to be inter-dependent (55). 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 nitrogen limitation of *Symbiodinium*.

Figure 3. Conceptual model of the role of coral-associated N\textsubscript{2} fixation in coral bleaching. In a pristine nutrient-poor environment, growth of algal symbionts (*Symbiodinium*) is regulated by the coral host via internal nitrogen (N) limitation. High seawater temperatures or dissolved organic carbon (DOC) levels stimulate nitrogenase activity and diazotroph proliferation, thereby increasing N\textsubscript{2} fixation activity in the holobiont. Excess fixed N is rapidly taken up by *Symbiodinium*, inducing nutrient balanced growth or even P starvation. This Phosphorus (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 would increase the susceptibility to photodamage due to a failure of host carbon concentration mechanisms (CCMs) causing CO2 limitation of photosynthetic dark reactions. In order to restore control over the symbiosis, we hypothesize the coral host would attempt to increase its P uptake and / or to remove and retain N from *Symbiodinium* in specialized structures of the host’s epidermal tissues.
Ecological relevance of elevated DOC levels on coral reefs

Coral reefs can be regionally exposed to periodically changing levels of DOC, and may therefore range from very low levels (reviewed in 36; 1-2 ppm in the current study) to exceeding 100 ppm (59–61)). The DOC enrichment in the present study achieved a >10-fold increase relative to the untreated ambient control, and therefore constitutes a realistic and ecologically relevant enrichment level.

It has to be noted however that the effects of DOC enrichment depend on a variety of other environmental factors. In the present study, DOC enrichment caused a dramatic significant shift in the N:P ratios of the two eukaryotic departments of the P. verrucosa holobiont: the host, and *Symbiodinium*. These changes were likely facilitated by the oligotrophic conditions of the Red Sea water used in this experiment. In a naturally less oligotrophic system like the Caribbean on the other hand, higher enrichment would likely be necessary to evoke similar responses. This being said, DOC additions in the same order of magnitude induced coral bleaching and mortality in previous studies from the Northern Gulf of Aqaba (62) and the Florida Keys (36, 63).

The ecological and physiological effects of DOC enrichment on coral holobionts will also depend on its quality and composition. Whilst a large fraction of DOC in the Caribbean may be constituted of refractory DOC from terrestrial inputs (64, 65), 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 will be different from those of refractory DOC. Labile DOC can be introduced onto coral reefs from various sources. Among these, municipal sewage and algal exudates contain a large fraction of labile DOC, and exhibit a similar neutral sugar composition as used in this study (66, 67). Indeed, macroalgae exudates differentially enrich and stimulate cell growth, favoring the prevalence of potentially pathogenic bacteria (67), and cause shifts towards less efficient copiotrophic reef bacterial communities (68, 69).
Coral reef resilience in a changing ocean

Coral-associated nitrogen fixation is increasingly recognized as beneficial for coral health (23) and fundamental for sustaining primary productivity under (seasonally) changing environmental conditions (28, 35). On the other hand, we show here that diazotrophs may also contribute to the destabilization of the coral-algae symbiosis, and thereby promote coral reef degradation (23). While the current study used DOC enrichment to induce bleaching, our findings are highly applicable to thermal bleaching as the same underlying microbial processes appear to be in place. This is evident from the strikingly similar increases in diazotroph abundances (34) and activity (35) in corals under heat stress or bleaching as well as high DOC provision. Thermal bleaching has long been recognized as one of the most severe threats to modern coral reefs (70). Our findings imply that the ubiquitous presence of diazotrophs in the microbiomes of most coral species may pose a threat to corals in a warming ocean (46). However, similar as in thermal bleaching (71), changes in the coral-associated nitrogen fixation activity and its impact on holobiont functioning will be largely dependent on the environmental (i.e., holobiont) context, for instance on the nutritional status or taxonomic affiliation of the coral host.

Reshef et al. (72) suggested that a restructuring of the coral microbiome may facilitate the rapid acclimatization of the coral holobiont to changing environmental conditions. Therefore, a reduction in diazotroph abundance could potentially enhance the thermal tolerance of corals. 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 global climate change outpaces the coral’s capacity for adaptation (73).

There may be no rapid solution to reduce the effects of global climate change in the near future; all the more important becomes mitigation, e.g. by reducing local anthropogenic stressors, which will be essential for future conservation efforts. We show here that DOC enrichment can become a dominant driver of reef degradation and may ultimately render corals more susceptible to the effects of climate change. Thus, we suggest local management efforts
should focus on the reduction of DOC input and loading on corals reefs. Sources of DOC enrichment on coral reefs include sewage and wastewater (74) as well as excessive algal abundance (37). Consequently, in order to reduce the amount of DOC to diminish microbially-driven reef degradation processes, management measures would benefit best from combined efforts (69, 75). We therefore suggest that improved wastewater treatment to effectively retain inorganic and organic nutrients (13, 76) and the restoration of herbivorous fish stocks to control harmful algal growth (77) would likely increase the resilience of corals to ocean warming.

MATERIAL AND METHODS

Experimental design, biological material, and treatments

The experiments were conducted at the wet lab 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, consisting of three replicate experimental tanks (100 L each) connected via a recirculation reservoir bin (100 l) containing heating equipment. Natural Red Sea reef water was circulated in each unit with thirty percent of the water 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 heat and light stress (seawater temperature at 27 °C, salinity at 40.5 PSU, photosynthetic active radiation 100 quanta μmol s⁻¹ m⁻² on a 12:12 h day/light cycle. Dissolved oxygen remained constantly > 6 mg l⁻¹ seawater in both treatments at all times; for details, see Supplementary Table S1). In three aquaria, labile DOC levels were manipulated by daily additions of a 10 mg l⁻¹ 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 (66) and coral reef macroalgae exudates (67). The other three aquaria were maintained at ambient DOC levels.

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 40x40 mm stone tiles with a two-part epoxy putty (ReefConstruct, AquaMedic, Germany). Coral fragments from all colonies were distributed evenly among the aquaria 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 one week prior and throughout the experiment to avoid confounding effects from additional nutrient uptake via heterotrophy.

A detailed description of all measured response parameters is provided in the Supplementary Information. Briefly, seawater samples were collected every 7 days, filtered and frozen for subsequent analysis of DOC, total dissolved nitrogen (TN), and phosphorus (TP) content. Samples were analyzed by the Marine Chemistry Lab at the University of Washington, USA, and the Analytical Core Lab (ACL) at KAUST, Saudi Arabia, respectively. Photosynthesis and respiration rates were quantified from start and endpoint O$_2$ measurements in gas-tight incubation chambers during dark and light incubations (32). Similarly, nitrogen fixation rates were indirectly quantified via measurements of ethylene (C$_2$H$_2$) evolution using the acetylene reduction assay (78). The maximum (or dark-adapted) quantum yield was measured with a submersible pulse amplitude modulation (PAM) fluorometer. For Symbiodinium density estimates, cells were extracted from coral tissue and counted using a haemocytometer (28). Stable N ($\delta^{15}$N) signatures and N:P ratios were determined from dried coral tissue and extracted Symbiodinium cells with an isotope ratio mass spectrometer and a photometer, respectively (26, 79). Changes in the overall bacterial community composition were determined by assessing 16S rRNA diversity by Next Generation Sequencing on the MiSeq Illumina platform. Changes in the relative abundance of coral-associated nitrogen fixing microbes were assessed by qPCR of the nifH gene, encoding for a subunit of the nitrogenase enzyme (80). All statistical analyzes are explained in detail in the Supplementary Information.
ACKNOWLEDGEMENTS

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REFERENCES


APPENDIX

Chapter 2

Supplementary Information
Supplementary Information Chapter 2:

Stimulated nitrogen fixation causes coral bleaching in the absence of heat and light stress

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$\dagger$ contributed equally

$\star$ contributed equally

Supplementary information
SUPPLEMENTARY RESULTS

**Supplementary Table S1.** Seawater maintenance conditions in experimental tanks for treatments over time. Data are presented as mean ± SE. DO = dissolved oxygen; DOC = dissolved organic carbon; ppm = part per million; TN = total nitrogen content; TP = total phosphorus content.

<table>
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<th>Days</th>
<th>Treatment</th>
<th>Temperature (T°C)</th>
<th>Salinity [PSU]</th>
<th>DO [mg O₂ l⁻¹]</th>
<th>TN [μM]</th>
<th>TP [μM]</th>
<th>DOC [ppm]</th>
</tr>
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<td>41.55 ± 0.04</td>
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<td>18.90 ± 0.94</td>
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<td>15.17 ± 0.80</td>
<td>0.22 ± 0.01</td>
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<td>0.19 ± 0.02</td>
<td>19.32 ± 0.03</td>
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**Supplementary Table S2.** Results of generalized linear models (GLMs) of seawater nutrient and dissolved organic carbon (DOC) content. TN = total nitrogen; TP = total phosphorus. All models based on gamma distribution and best fitting link function.

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**Supplementary Table S3.** Results of generalized estimating equations (geglms)* and generalized linear models (glms)** of individual response parameters. All models based on gamma distribution and best fitting link function.

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<td>treatment x time x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>compartment</td>
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<td>48</td>
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</tr>
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<td></td>
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<td>1</td>
<td>48</td>
<td>0.306</td>
</tr>
<tr>
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<td>1</td>
<td>48</td>
<td>0.041</td>
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<tr>
<td>treatment x time x</td>
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<td></td>
<td></td>
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<td>compartment</td>
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<td>1</td>
<td>48</td>
<td>0.346</td>
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</table>
**Supplementary Table S4.** Summary of alpha diversity indices (means ± SE) of bacterial communities associated with the *Pocillopora verrucosa* holobiont in two treatments over time. The coverage was > 99.998 in all samples.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Nu. Of sequences</th>
<th>Coverage</th>
<th>Chao</th>
<th>Inverse Simpson Index</th>
<th>Simpson Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>control</td>
<td>98494 ± 27444</td>
<td>1.00</td>
<td>468.7 ± 104.7</td>
<td>0.006 ± 0.000</td>
<td>2.11 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>DOC</td>
<td>116230 ± 28495</td>
<td>1.00</td>
<td>478.2 ± 35.3</td>
<td>0.007 ± 0.003</td>
<td>2.23 ± 0.49</td>
</tr>
<tr>
<td>7</td>
<td>control</td>
<td>138866 ± 25698</td>
<td>1.00</td>
<td>295.5 ± 116.3</td>
<td>0.011 ± 0.002</td>
<td>1.86 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>DOC</td>
<td>165177 ± 38011</td>
<td>1.00</td>
<td>227.2 ± 62.6</td>
<td>0.014 ± 0.008</td>
<td>1.55 ± 0.30</td>
</tr>
<tr>
<td>14</td>
<td>control</td>
<td>112061 ± 12852</td>
<td>1.00</td>
<td>369.9 ± 30.4</td>
<td>0.009 ± 0.002</td>
<td>1.86 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>DOC</td>
<td>145226 ± 29028</td>
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<td>366.1 ± 31.3</td>
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<tr>
<td>28</td>
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<td>144268 ± 28704</td>
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<td>0.012 ± 0.002</td>
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</tr>
<tr>
<td></td>
<td>DOC</td>
<td>247890 ± 24850</td>
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<td>243.1 ± 54.5</td>
<td>0.011 ± 0.003</td>
<td>1.55 ± 0.32</td>
</tr>
</tbody>
</table>
Supplementary Table S5. Results of analysis of molecular variance (amova) of overall bacterial 16S rRNA sequences over both treatments and all sampling points. Significant differences are presented in bold italic. 0, 7, 14, 28 = duration under exposure to treatment (in days).

<table>
<thead>
<tr>
<th>time</th>
<th>Group</th>
<th>Among</th>
<th>Within</th>
<th>Total</th>
<th>Fs</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
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<td>SS</td>
<td>0.465</td>
<td>1.367</td>
<td>1.833</td>
<td>2.268</td>
<td>0.033*</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>MS</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0.769</td>
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<tr>
<td></td>
<td>dF</td>
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<td>10</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MS</td>
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<td>0.077</td>
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<tr>
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<tr>
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<td>10</td>
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<th>Within</th>
<th>Total</th>
<th>Fs</th>
<th>p</th>
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</thead>
<tbody>
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<td>MS</td>
<td>0.145</td>
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</table>

<table>
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<tr>
<th>treatment * time</th>
<th>Group</th>
<th>Among</th>
<th>Within</th>
<th>Total</th>
<th>Fs</th>
<th>p</th>
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<tbody>
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**Supplementary Table S6.** OTU abundance table of the coral-associated bacterial community associated with *P. verrucosa* (shortened; the entire community encompasses 1367 OTUs). Presented are mean numbers of sequences of the five most abundant OTUs (01 – 05) as well as all Vibrionaceae. C = control treatment; T = high DOC treatment.

*note: this is a shortened (only 5 most abundant coral-associated bacterial OTUs plus members of the family Vibrionaceae displayed) and simplified version of the original table to accommodate the requirements for printing and online formatting of this thesis. The original version of this table will be published as an excel sheet containing all bacterial OTUs and the full taxonomic annotation and representative sequence in the online Supplementary Information of this publication.*

<table>
<thead>
<tr>
<th>Otu</th>
<th>C0</th>
<th>T0</th>
<th>C7</th>
<th>T7</th>
<th>C14</th>
<th>T14</th>
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<th>T28</th>
<th>Total # Seqs</th>
<th>Annotation</th>
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<tr>
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<tr>
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<td>0</td>
<td>1</td>
<td>Gammaproteobacteria_Vibrionales_unclassified Vibriaceae</td>
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</tbody>
</table>
Supplementary Figure S1. Bacterial community composition associated with the tissues of *P. verrucosa* from control and under elevated labile dissolved organic carbon (DOC). Inner circles represent community composition on the class level, outer circles represent the operational taxonomic unit (OTU) level. All data are presented as average based on *n*=3 replicates. For full amova statistics, see Supplementary Table S5.
Bacterial community

For the bacterial community associated with the tissues of *P. verrucosa*, we generated a total of 24 16S rRNA gene libraries, comprising a total of 6474720 sequences (distributed over 3 coral replicates x 2 treatments x 4 time points = 24 in total). After removing chimeras, chloroplast, mitochondria, archaea, eukaryotic sequences, a total of 3504646 reads with an average length of 293 bp were retained for further analyses. Classification against the 2015 version of the Greengenes database (94, 95) yielded a total of 1367 distinct OTUs. The overall bacterial community exhibited no treatment specific changes in composition, but showed a loss in diversity over time for both treatments (Supplementary Table S4, S5). Overall, the community was dominated by Gammaproteobacteria (78 – 85 % of all sequences) and Chlamydiia (4 – 20 % of sequences). The class Gammaproteobacteria was mainly comprised by two distinct Endozoicomonaceae genotypes (OUT 1 & 3) (Supplementary Figure 1). Members of opportunistic and potentially pathogenic families such as Vibrionaceae, Rhodobacteraceae, Bacteriovoraceae, or Flavobacteraceae were identified as part of the rare fraction of the microbiome, yet showed no treatment specific increases.

SUPPLEMENTARY METHODS

Sampling

Nitrogen fixation activity, diazotroph abundance, maximum quantum yield and *Symbiodinium* density were measured at day 0, 7, 12 and 28 of the experiment. Remaining response parameters were measured at the first and last day of the experiment. Non-invasive parameters were addressed in a repeated measure design to increase statistical power. For the invasive response parameters, single fragments originating from all mother colonies and treatments were rinsed with filter-sterilized seawater (FSW; 0.22 μm) and flash-frozen in liquid N₂ and stored at -80°C until further processing. All sampling and measurements were conducted in the morning (8 am – 10 am), except for N₂ fixation activity (continuous measurement over the course of 24 h) and maximum quantum yield (1 h into the dark phase).
**Oxygen (O₂) and ethylene (C₂H₄) evolution**

Gross photosynthetic (P₇₀), net photosynthetic (P₅₇) and dark respiration (R; calculated via O₂ evolution), and N₂ fixation rates (calculated indirectly from C₂H₂ reduction assay) were quantified during incubations in gas-tight 1 L glass chambers. Six fragments per treatment were used for repeated O₂ evolution and N₂ fixation measurements respectively, resulting in a total number of 24 fragments. Four additional chambers per treatment filled exclusively with treatment water served as controls to account for planktonic background metabolism. During incubations, chambers were submersed in a water bath to maintain equal temperature, and constantly stirred (600 rpm). All rates were corrected for the respective mean seawater control signals and normalized to incubation time and coral surface area, which was quantified based on 3D models using the digital modeling applications 123D Catch and Meshmixer (Autodesk Inc., USA) (81).

Oxygen evolution was based on the difference of O₂ concentrations at the start and end of the same incubations during light (P₅₇) and dark (R) with a salinity-corrected optode sensor (FDO®925 – Optical Dissolved Oxygen Sensor, MultiLine® IDS 3440, WTW GmbH, Germany). Incubations lasted ~2 h each to avoid supersaturation/depletion of O₂, respectively. Gross photosynthesis was calculated based on P₇₀ = P₅₇ + R.

Nitrogen fixation rates were quantified indirectly from ethylene (C₂H₄) evolution rates from acetylene (C₂H₂) reduction assays without conversion into actual fixation rates, as we acknowledge the ongoing discussion about the correct conversion factor (78, 82). Corals were incubated for 24 h in chambers containing 800 mL of the seawater (10 % previously saturated with C₂H₂) and a 200 mL headspace (ambient air enriched with 10 % C₂H₂). 1 mL gas samples were collected at 0, 12, and 24 h of incubation. Nitrogen fixation rates were calculated based on C₂H₄ concentration differences between time intervals from 0 to 12 h (dark phase) and 12 to 24 h (light phase) (83). C₂H₄ concentrations in gas samples were quantified by gas chromatography (Varian 3800 with AL203/KCL 50 x 0.53 mm column and flame ionization detector).
Measurement of maximum PSII quantum yield (PAM fluorometry)

Photosynthetic activity of *Symbiodinium* cells *in hospite* was confirmed by measuring PSII maximum quantum yield ($F_v/F_m$) of dark-adapted coral fragments (12 per treatment) 1 h into the 12 h dark phase. Measurements were carried out using a pulse amplitude modulation (PAM) fluorometer (DIVING-PAM, Walz, Germany).

*Symbiodinium density*

*Symbiodinium* cells were freshly isolated from coral tissue by NaOH extraction (84). Subsamples of individual coral fragments were incubated in 1M NaOH at room temperature and frequently and vigorously shaken. After 1 h, the skeleton was removed. Suspended *Symbiodinium* cells were spun down in a bench-top centrifuge for 5 min at 3,000 RCF, the supernatant discarded, and the *Symbiodinium* pellet washed 2 x in 1 ml PBS (1x). Subsequently, the pellet was resuspended in a 10 % PBS-buffered formaldehyde solution and stored at 4°C until further processing. *Symbiodinium* density was determined using a haemocytometer (28).

*Elemental analyses*

Individual coral branches were air-blasted with FSW (0.22 μm) and the resulting tissue slurry homogenized for 30 s at 3,500 rpm with an UltraTurrax (T 18 basic, IKA, Germany). Homogenized slurry was separated into *Symbiodinium* cells and coral tissue by two steps of centrifugation (5 min at 3,000 rcf) and resuspension in FSW. Tissue and resuspended *Symbiodinium* cells were separately filtered onto pre-weighed and pre-combusted GF-Fs (47 mm, Whatman, GE Healthcare, Germany) and subsequently dried at 60 °C until constant weight. Dried samples were directly measured ($^{15}$N) with a EuroVector elemental analyzer (EURO EA 3000). Nitrogen contents were calculated using elemental standards (OAS; analytical precision ≤0.1% of the standard value). Isotopic analysis of $\delta^{15}$N signatures of dried material relative to atmospheric N was run with an isotope ratio mass spectrometer (Finnigan Corp., San Jose, CA). Isotopic values are expressed as either enriched or depleted in $\delta^{15}$N. Average reproducibility
Based on replicate measurements of stable N isotopes are usually about 0.15 ‰ (85). Analyses of total nitrogen to phosphorus ratios (N:P ratios) of coral tissue and Symbiodinium cells were conducted photometrically (Spectroquant ® Pharo 300 UV/VIS Spectrophotometer, Merck, Germany) from samples extracted from non-acidified GF-Fs using a modified protocol for marine and brackish seawater samples (79). Total Nitrogen and Phosphorus were measured against standard calibrations at 540 and 880 nm, respectively.

**Bacterial community composition and diazotroph abundance**

DNA of coral tissue-associated bacteria was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer’s instructions. Coral tissue was air-blasted from the skeleton with AP-1 tissue lysis buffer. 200 μl tissue slurry in 200 μl AP-1 were used for DNA extraction. Extracted DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, USA) and adjusted to a concentration of 10 ng μl⁻¹.

The overall bacterial community composition was determined from 16S rRNA diversity by Next Generation Sequencing on the MiSeq Illumina Platform. PCR amplifications were performed in triplicate reactions with Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany)All primers contained Illumina adapter overhangs (underlined below; Illumina, San Diego, CA, USA). For amplicon-specific PCRs of the eubacterial community, we used the primers 16SMiSeqF-Andersson 5´-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGG ATT AGA TAC CCT GGT-3´ and 16SMiSeqR-Andersson 5´-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCR RCA CGA GCT GAC GAC-3´ that target the variable regions 5 and 6 of the 16S rRNA gene (86). For individual PCR reactions, DNA concentrations were aliquoted to 10 ng/μL, with 10 μL Qiagen Mix, 0.5 μL of each 10 μM primer mix, 1 μL of DNA template, and RNAse-free water to adjust the reaction volume to 20 μl. The thermal profile for the PCRs was as follows: 94°C for 15 min, then 35 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 30 s, followed by one cycle of 72°C for 10 min and hold at 4°C; and 95°C for 15 min, followed by 27 cycles of 95°C for 40 s, 55°C for 40 s, 72°C for 40 s, and a final extension cycle of 72°C at 10 min. 10 μl of each PCR product was run on
an 1% agarose gel to visualize successful amplification. Sample triplicates were subsequently pooled and then purified using the Agencourt AMPure XP magnetic bead system (Beckman Coulter, Brea, CA, USA). Purified PCR products were subjected to an indexing PCR to add Nextera XT indexing and sequencing adapters (Illumina) according to the manufacturer’s protocol. Indexed products were again purified, quantified on the BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and QuBit (Quant-IT dsDNA Broad Range Assay Kit; Invitrogen, Carlsbad, CA, USA), and pooled in equimolar ratios. The final pooled library was purified on a 2% agarose gel to remove excess primer dimer. The library was sequenced at 8pM with 10% phiX on the Illumina Miseq, 2x 300 bp end version 3 chemistry according to the manufacturer’s specifications at the Bioscience Core lab (KAUST, Saudi Arabia).

The determination of absolute and relative gene-copy numbers of the target genes 16S rRNA and nifH was accomplished with qPCR with the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA) according to manufacturer’s instructions. Amplifications were performed in triplicate reactions with the Platinum SYBR Green qPCR SuperMix kit (Invitrogen, Carlsbad, CA, US), with 10 μL SuperMix, 0.4 μL ROX reference dye, 0.4 μL of each 10 μM primer, 1 μL of input DNA template, and RNase-free water to adjust the reaction volume to 20 μl. To amplify the bacterial 16S rRNA gene, again the the primers 781F 5´-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG -3´ and 1061R 5´-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA -3´ were used (86). For amplification of nifH, the primers F2 5´-TGYGAYCCIAAIGCIGA -3´ and R6 5´-TCIGGIGARATGATGGC -3´ were used (80). The qPCR was run under the following thermal profile: 2 min at 50 °C, 1 min at 94°C, followed by 50 cycles of 94°C for 30 s, 51°C for 1 min, 72°C for 1 min, and an extension cycle of 1 min at 72°C. The specificity of the amplifications was confirmed by melting curve analysis. Standard calibration curves were run simultaneously covering 7 orders of magnitude (10⁴ – 10⁹ copies of template per assay for 16S rRNA and nifH gene, respectively). The qPCR efficiency (E) was > 85 % for both primers, calculated according to the equation E=[10 (-1/slope)-1]. Relative fold change of nifH gene copy numbers was calculated as 2(-ΔΔCt) using day 0 control samples as reference.
Seawater nutrient measurements

Treatment water samples for nutrient analysis were collected at all sampling points. Treatment water was sampled in 30 ml 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 dissolved organic carbon was performed with a Apollo 9000 Total Organic Carbon (TOC) Analyzer™ (Teledyne Instruments Tekmar, USA), and total nitrogen and phosphorus concentrations were simultaneously measured according to standard method (SM) 4500-P J (87).

Statistical analysis

For statistical analysis, univariate general linear models were fitted to our data in R v3.2.2 (88). N₂ 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 (89). 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 analysed 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. All data are reported as mean ± SE, asterisks indicate statistically significant differences (* P < 0.05, ** P < 0.01)

Bacterial community analysis

For amplicon analysis of coral tissue-associated eubacterial communities, mothur v1.36.1 was used (90). Briefly, reads were demultiplexed, and sequences were quality trimmed and pre-clustered (2 bp difference) (91). Sequence reads were split according to barcodes, assembled to contigs, and quality trimmed. Identical sequences (duplicates) were merged using the
unique.seqs command, and count.seqs was used to calculate the number of sequences over samples represented by the remaining representative sequence. Next, singletons and rare sequences (n < 10 over all samples) were removed. Remaining sequences aligned against the SILVA database (release 119; 67). Chimeric sequences were removed using the UCHIME command (93) and reads assigned to chloroplasts, mitochondria, archaea, and eukaryotes were excluded. Sequences were classified into operational taxonomic units (OTUs) against Greengenes database (release gg_13_8_99; bootstrap = 60; 97 % similarity cut-off chosen to obtain OTUs; 69). Bacterial community composition pie charts were created on the OTU and class level using the means of relative abundances from replicates (n = 4). Alpha diversity measures (number of OTUs, Chao estimate of species richness, inverse Simpson index, Simpson evenness) and an analysis of molecular variance (amova) were subsequently calculated as implemented in mothur.
SUPPLEMENTARY REFERENCES


CHAPTER 3

Do differences in microbial nitrogen fixation align with differential stress susceptibility in reef-building corals?

Pogoreutz, C., Rädecker, N., Cárdenas, A., Gärdes, A., Wild, C., Voolstra, C. Differences in microbial nitrogen fixation align with stress susceptibility in reef-building corals. This chapter is in preparation for submission as a short communication to *Environmental Microbiology.*
ABSTRACT

Microbial nitrogen fixation (diazotrophy) is a functional trait widely associated with tropical scleractinian corals. While beneficial for coral holobiont productivity under undisturbed conditions, it may increase the susceptibility of corals to the effects of global environmental change. Here we provide a comparative analysis of the molecular and physiological basis of diazotrophy in two widely distributed Indo-Pacific coral families, the Pocilloporidae and Fungiidae. High diazotroph abundance and nifH gene expression along with noticeable nitrogen fixation activity ($0.13 \pm 0.04 \text{ nmol } C_2H_4 \text{ cm}^{-2} \text{ h}^{-1}$ averaged over 24 h) were associated with the Pocilloporidae. In contrast, diazotroph numbers and gene expression were two orders of magnitude lower in the Fungiidae (20 – 100 and 430 – 480 fold lower respectively than in *Pocillopora verrucosa* and *Stylophora pistillata*). Under environmental stress (soft coral and algae exudate provision), Fungiidae-associated diazotrophs exhibited elevated abundances and nifH gene expression compared to control treatments, but nitrogen fixation activity was still not detectable in this group. While more work is needed at this point, our data suggest a potential link between diazotrophy with coral stress susceptibility, indicating that the dependence on nitrogen fixation could decrease coral resilience under global environmental change.
Tropical scleractinian corals are holobionts consisting of the coral animal (or host), dinoflagellate algae of the genus *Symbiodinium*, and a diverse assemblage of other microbes (1). These microbes form host-specific associations and provide key functional traits within the coral holobiont. Among these traits, biological nitrogen fixation is of high ecological significance, and nitrogen fixers (diazotrophs) are diversely and ubiquitously associated with tropical corals (2, 3). As biologically fixed nitrogen is readily taken up by *Symbiodinium* (4), it helps sustain high holobiont productivity when nutrients are scarce (5). This marks one of several remarkable adaptations of coral holobionts to the highly oligotrophic tropical waters in which they thrive in (6). In this context, nitrogen is a limiting nutrient, and the coral-algae symbiosis is strictly regulated by the coral host via nitrogen limitation to *Symbiodinium* (7–9). Therefore, the overstimulation of coral-associated nitrogen fixation activity and the subsequent excess availability of ‘new’ fixed nitrogen are likely to disrupt holobiont functioning (10). While there is recent evidence for this scenario (11; Pogoreutz et al. in prep.), the molecular and physiological basis of nitrogen fixation in corals is poorly understood. Therefore, in this study, we compared diazotroph abundance and *nifH* gene expression with nitrogen fixation activity in a comparative coral taxonomic framework covering four common coral species from two widely distributed families (see below for further details) to identify a putative link between diazotrophy and stress susceptibility.

We assessed tissue-associated relative gene copy numbers and expression rates of *nifH*, a common biomarker for diazotrophs (12), in a quantitative PCR (qPCR) approach for the Indo-Pacific coral families Pocilloporidae (i.e., *Pocillopora verrucosa*, *Stylophora pistillata*) and Fungiidae (i.e., *Pleuractis granulosa*, *Ctenactis echinata*). *nifH* qPCR data were normalized against *Symbiodinium* abundance via the Internal Transcriber Spacer 2 (ITS2) genetic region as *Symbiodinium* is the main sink for fixed nitrogen within the coral holobiont (4, 13), and *Symbiodinium* growth rates correlate with diazotroph abundances in corals (14) (see Supplementary Methods: Normalization of *nifH* qPCR data). To confirm active *nifH* transcription,
we measured nitrogen fixation rates via ethylene (C\textsubscript{2}H\textsubscript{4}) evolution in *P. verrucosa* and *P. granulosa* in acetylene reduction assays over 24 h.

Additionally, nitrogen fixation was quantified for stressed *P. granulosa* under high concentrations of macroalgae and soft coral exudates. These exudates are known to detrimentally affect scleractinian corals. Particularly macroalgae exudates can induce coral bleaching, disease, and mortality (15–18), effects which were recently attributed to coral-associated microbial community shifts (19, 20), increased bacterial growth rates, and respiration (15, 17), and the subsequent formation of hypoxic layers on the coral surface (17). These dramatic responses of coral-associated microbial communities and subsequent (sub)lethal responses of the coral holobiont were proposed consequences of high contents of allelochemicals (21–23) as well as dissolved organic matter (DOM), more specifically dissolved organic carbon (DOC). Consequently, we selected soft coral and macroalgal exudates due to their high DOM content (24–27) and high carbon to nitrogen ratios (i.e., high carbon but low nitrogen content) (26), and therefore expected to have stimulative rather than inhibitory effects on diazotrophy.

In contrast to the normalization against the abundance of the eubacterial 16S rRNA gene, normalization against ITS2 reflected the measured nitrogen fixation rates in the corals *P. verrucosa* and *P. granulosa*. Corals in the Pocilloporidae family exhibited high *nifH* gene copy numbers and expression rates. Simultaneously, nitrogen fixation activity measured in *P. verrucosa* was well within in the range reported previously (0.13 ± 0.04 nmol C\textsubscript{2}H\textsubscript{4} cm\textsuperscript{-2} h\textsuperscript{-1} averaged over 24 h) (5). In contrast, relative diazotroph abundance and *nifH* gene expression remained up to two orders of magnitude lower in the Fungiidae (20 – 100 and 430 – 480 fold, respectively). Concomitantly, no nitrogen fixation activity was detectable in *P. granulosa*. After 24 h of high DOM provision, *P. granulosa* remained visibly healthy, and no nitrogen fixation activity was detectable (detection limit: 0.5 ppm C\textsubscript{2}H\textsubscript{4}). However, relative diazotroph abundance and *nifH* gene expression rapidly increased within 24 h (Figures 1A,B; Supplementary Table 3A-C). We argue that the absence of measurable nitrogen fixation activity despite the increases in
diazotroph abundance and \textit{nifH} gene expression is due to the inherently low initial \textit{nifH} abundance and expression associated with \textit{P. granulosa}. Nonlinear regression further revealed a strong relationship between \textit{nifH} copy numbers and gene expression in the two species of Pocilloporidae (R² = 71.49, p = 0.0339), but only a weak relationship in the two Fungiidae (R² = 0.07, p = 0.0288; Supplementary Table 3D-E), suggesting that its minute diazotroph community may not be fixing nitrogen at ecologically significant rates. While Pocilloporidae presumably strongly depend on biological nitrogen fixation to supplement nitrogen acquisition (5), the low diazotroph activity in Fungiidae suggests the efficient exploitation of other means of nitrogen acquisition to supplement its metabolic requirements. Similarly, Shashar et al. (28) reported nitrogen fixation activity in \textit{Pocillopora damicornis} and \textit{Stylophora pistillata}, yet a lack of nitrogen fixation in \textit{Fungia fungites}. The absence of nitrogen fixation may therefore widely apply to the family Fungiidae. While at this point more data are needed, we propose that these marked patterns in diazotrophy on the coral family level could potentially be related to differential heterotrophic feeding capacities. Whilst Fungiidae exploit a large variety of food sources (29), Pocilloporidae exhibit a rather limited heterotrophic capacity and, thus, may rather depend on nitrogen fixation to supplement their metabolism (30). Therefore, identifying potential linkages between heterotrophic capacity and the ecological reliance on nitrogen fixation activity in coral holobionts may be an interesting new avenue of research.

Importantly, the reported differences in nitrogen fixation and diazotroph abundance reported here coincide with differential stress susceptibility in the two coral families. Pocilloporidae are highly sensitive to thermal stress (31–33). In contrast, Fungiidae are less susceptible to ocean warming (31, 34), and highly resistant to ocean acidification (35), sedimentation (36), elevated salinity (37), and fragmentation (38). Fungiidae further are efficiently feeding single polyps. In combination with potentially being able to store large energy reserves in their fleshy tissues (37), reducing the metabolic need for nitrogen fixation may increase their overall stress resistance. We argue that dependence on microbial nitrogen fixation may render corals more susceptible to environmental stressors, as stimulation of this functional
trait can rapidly derail the coral-algae symbiosis via excess nitrogen availability (10; Pogoreutz et al. in prep.). In consequence, nitrogen fixation could become an Achilles’ heel of scleractinian holobionts in a time of anthropogenically-driven environmental change. As other nitrogen fixing corals however, for instance *Montastrea cavernosa* appear to be quite resistant to the effects of global environmental change (31), more in-depth research efforts are required to delve deeper into this issue, taking the environmental, ecological, and taxonomic context as well as different *Symbiodinium* associations of different coral holobionts into account.
Figure 1. Relative nifH gene copy numbers and expression rates associated with tropical scleractinian coral tissues (fold change; referenced against the Internal Transcriber Space 2, or ITS2). A. Comparative species framework: Fungiidae (Pleuractis granulosa, Ctenactis echinata) vs. Pocilloporidae (Pocillopora verrucosa, Stylophora pistillata). Significant differences are apparent across all samples and treatments for nifH gene copy numbers (Kruskal-Wallis Test, $H = 15.316, df = 5, p = 0.009$) and the coral family level (one-way ANOVA, $F = 40.679, p < 0.001$), respectively and for nifH gene expression (one-way ANOVA, $F = 12.298, p < 0.001$) and on the coral family level (one-way ANOVA, $F = 22.179, p < 0.001$), respectively. B. The fungiid coral Pleuractis granulosa under different treatments of high dissolved organic matter (DOM) availability. Asterisks denote significant differences based on ANOVA and multiple comparisons.
(see Supplemental Information for details), specifically between Fungiidae vs. Pocilloporidae (A) and high DOM treatments vs. *P. granulosa* (con) (B). Con = control; caul = Macroalgal (*Caulerpa*) exudate; xen = Soft coral (*Xenia*) exudate.

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REFERENCES


APPENDIX

Chapter 3

Supplementary Information
Supplementary Information Chapter 3:

Do differences in microbial nitrogen fixation align with differential stress susceptibility in reef-building corals?

Claudia Pogoreutz¹²³†, Nils Rädecker¹³†, Anny Cárdenas¹³⁴, Astrid Gärdes⁴, Christian Wild¹²*, Christian R. Voolstra³*

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Supplementary Information
SUPPLEMENTARY METHODS

Collection and Maintenance of Corals

Four species of coral, i.e. two species each of Fungiidae (*Pleuractis granulosa, Ctenactis echinata*) and Pocilloporidae (*Pocillopora verrucosa, Stylophora pistillata*) were collected at the exposed site of the inshore reef Fsar (22° 13.974N, 39° 01.760E) off the Saudi Arabian coastline in the Central Red Sea. The Saudi Coastguard Authority under the auspices of KAUST University issued sailing permits to the collection site, which included sampling of coral specimens.

For both, nitrogen fixation measurements and RNA extractions, a total of 18 individuals of the common coral *Pleuractis granulosa* (formerly *Fungia granulosa*; 1)) and triplicate samples of *Pocillopora verrucosa* were collected (see Supplementary Table 1). Additionally, triplicate samples of the fungiid and pocilloporid corals *Ctenactis echinata* and *Stylophora pistillata* were collected for RNA extraction (for overview, see Supplementary Table 1 for details). The collection depth for all corals was 5 – 6 m, at an irradiance of approx. 1000 quanta μmol s⁻¹ m⁻². Immediately upon collection in the field, corals of all four species were flash-frozen in liquid nitrogen (collection and freezing from 9 – 10 am). All flash-frozen corals were stored at -80°C until further processing. Corals intended for nitrogen fixation measurements (ARA incubations) were immediately transferred to the wet lab facility of the Coastal and Marine Resources Core Lab at the King Abdullah University of Science and Technology (KAUST), KSA.

Corals were acclimated for four weeks prior to the start of the experiment in separate aquarium tanks with circulating coral reef water under constant maintenance conditions (seawater temperature at ~28 °C; salinity at ~40 PSU; dissolved oxygen > 6.0 mg O₂ l⁻¹, total nitrogen ≤ 20 μMol l⁻¹, and phosphorous ≤ 0.3 μMol l⁻¹ at all times; and a photon flux of ~100 μmol m⁻² s⁻¹ on a 12:12 h daylight cycle; temperature). The system was comprised of two identical units including heating equipment and life support systems. Thirty per cent of the water was exchanged on a daily basis, and freshwater added manually to maintain salinity.
Exudation Assay

In order to stimulate nitrogen fixation activity via the provision of labile dissolved organic matter (DOM; 2, Pogoreutz et al. submitted), we harvested mucus and exudates from soft corals and macroalgae collected at a nearby reef (see below), which are rich in labile dissolved organic carbon and stimulate coral-associated heterotrophic microbial activity (3–7). While these exudates may also contain nitrogenous compounds, the relative contribution of (organic) nitrogen is orders of magnitude lower than that of organic carbon (4, 5). Therefore, we assumed no inhibitory effects of organic nitrogen availability by exudate treatments. For this purpose, we collected several colonies of the soft coral *Xenia* sp. and thalli of the green alga *Caulerpa racemosa*, total amounts covering about ~0.25 m² of benthic substrate. The organisms were collected at shallow depths (< 5 m for *Xenia*, < 1 m for *C. racemosa*) from the mid-shore reef al Fahal (22° 18.333N, 38° 57.768E) and the sheltered KAUST South Beach (22° 17.321N, 39° 05.217E), respectively. Individual thalli and colonies of *C. racemosa* and *Xenia* spp. respectively were left for 24 h to heal in aerated natural seawater at ambient temperature levels to prevent the release of cellular DOM during exudation (8). During the exudation, soft corals and algae were placed in untreated reef water in separate 1 l glass beakers (n = 6 each) for the duration of one daylight cycle (7 am – 5 pm) to maximize exudation. At the end of the exudation, crude exudates were pooled into one soft coral and one algal exudate stock, respectively. Pooled exudates and untreated control seawater were pre-filtered with 0.7 μm glass-fiber filters to remove coarse particulate matter and subsequently filter-sterilized at 0.22 μm. Triplicate subsamples of filter-sterilized exudates for measurement of respective DOM-contents were acidified with phosphoric acid until pH 2 and stored at -20°C until further processing. Finally, DOM measurements were conducted with diluted samples with an Apollo 9000 Total Organic Carbon (TOC) Analyzer™ (Teledyne Instruments Tekmar, Mason, Ohio) at the Analytical Core Lab at KAUST. The DOM measurements revealed final concentrations of 1 mgL⁻¹ for ambient
seawater (untreated controls) and 4 and 40 mgL$^{-1}$ in the algal and soft coral exudates, respectively.

**Nitrogen Fixation Measurements**

Nitrogen fixation rates were calculated indirectly from ethylene (C$_2$H$_4$) evolution via acetylene reduction assay (ARA) from air samples enriched in acetylene, as described by Rädecker et al. (9). Incubations were conducted in gas-tight 1 L glass chambers in 800 ml of treatment seawater (i.e., untreated seawater for controls and undiluted DOM-rich exudates) and 200 ml of 20 % acetylene-enriched air headspace. Six fragments of *P. verrucosa* and six individuals of *P. granulosa* were incubated in untreated seawater. Additionally, for each DOM treatment (i.e., provision of soft coral exudates and algal exudates), six individuals of *P. granulosa* were incubated (see Supplementary Table 1). For the incubations, unmanipulated seawater (ambient control) and the undiluted algal and soft coral exudates (DOM concentrations: 1, 4, and 40 mgL$^{-1}$, respectively) were used. Three additional chambers per treatment filled exclusively with treatment water served as controls for planktonic background metabolism. During the 24 h incubations, chambers were submersed in a heated water bath and constantly stirred (600 rpm) to ensure stable measurement conditions (28˚C, 12:12 h light/dark cycle, ~100 mol quanta μmol s$^{-1}$ m$^{-2}$). Gas samples were collected at 0 and 24 h of incubation. C$_2$H$_4$ concentrations in gas samples were quantified by gas chromatography (Varian CP-3800 GC with AL203/KCL 50 x 0.53 mm column and flame ionization detector; lower detection limit for C$_2$H$_4$ = 0.6 ppm). C$_2$H$_4$ contents in treatment water controls averaged about ~1 ppm (equalling 0.62 nm C$_2$H$_4$ l$^{-1}$ h$^{-1}$), and were used as a blank to correct for planktonic background metabolism of coral-associated nitrogen fixation rates. Nitrogen fixation rates are presented as C$_2$H$_4$ evolution rates without actual conversion into nitrogen fixation rates, as we acknowledge the ongoing discussion about the current conversion (10, 11). Data are presented as mean ± SE.

*Coral Tissue-Associated Relative nifH Gene Copy Numbers and Expression*
The tissue-associated relative gene copy numbers and expression rates of the *nifH* gene were quantified in the two Pocilloporidae (*P. verrucosa, S. pistillata*) and the two Fungiidae (*P. granulosa, C. echinata*), as well as *P. granulosa* from control and high DOM-treatments (Supplementary Table 1). Immediately upon collection in the field, corals of all four species were flash-frozen in liquid nitrogen (collection and freezing from 9 – 10 am). Corals subjected to ARA incubations were immediately flash-frozen in liquid nitrogen at the end of the incubation (around 9 am). All flash-frozen corals were stored at -80°C until further processing. RNA and DNA were extracted from all corals using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Germany) according to manufacturer’s instructions. Coral tissue was air-blasted off the skeleton on ice with RLT Plus buffer and using airflow from a sterile air gun. For *P. granulosa* and *C. echinata*, tissue was blasted off from both oral and aboral surfaces and pooled. 600 μl tissue slurry were used for RNA and DNA extraction. Extracts were quantified and quality checked using a NanoDrop 200c (Thermo Scientific, USA) and adjusted to a concentration of 10 ng μl⁻¹.

For cDNA synthesis for the qPCR assay, the SuperScript III First Strand Synthesis SuperMix kit (Thermo Scientific, USA) was used according to manufacturer’s instructions.

The determination of the relative gene copy numbers (from DNA) and expressed mRNA of *nifH* in coral tissue was accomplished by normalization against two reference genes and run simultaneously: the variable regions v5 and 6 of the 16S rRNA gene, a universal eubacterial marker (Andersson et al. 2008), and the ribosomal Internal Transcriber Spacer 2 region (ITS2), a multicycopy genetic marker to assess *Symbiodinium* diversity in corals (12). qPCR were run in triplicates with the Platinum SYBR Green qPCR SuperMix kit (Invitrogen, Carlsbad, CA, US) using 5 μL of SuperMix, 0.2 μL of ROX reference dye, 0.2 μL of each 10 μM primer, 1 μL of input cDNA, and RNAse-free water to adjust the reaction volume to 10 μl. For amplification of *nifH*, the primers F2 5´-TGYGAYCCIAAIGCIGA -3´ and R6 5´- TCIGGIGARATGATGGC -3´ were used (Gaby & Buckley 2012). For the amplification of the 16S rRNA gene, we used the primer pair 781F 5´- TCGTCCGACGGCTCACAGTGTATAAGAGACAG -3´ and 1061R 5´- GTCTCGTGGGCTCGAGATGTGTAATAAGAGACAG -3´ (Andersson et al. 2008). To amplify the
Symbiodinium ITS2 region, the primers ITSintfor2 5’- GAATTGCAGAATCGTG -3’ and ITS2-reverse 5’- GGGATCCATGCTTAAGTTCGCGGT -3’ were used (13). The qPCR was run under the following thermal profile: 2 min at 50 °C, 1 min at 94°C, followed by 50 cycles of 94°C for 30 s, 51°C for 1 min, 72°C for 1 min, and an extension cycle of 1 min at 72°C. The specificity of amplifications was confirmed by melting curve analysis. Standard calibration curves were run simultaneously covering 6 orders of magnitude (10^4 – 10^9 copies of template per assay). Relative fold change of nifH gene copy numbers was calculated as 2^{(ΔΔCT)} against each housekeeping gene using day 0 control samples as reference.

To test for statistically significant differences between treatments and coral species, variance analyses (detailed below) were run in SigmaPlot 13 (Systat Software GmbH, Germany) on the ΔCt values (Supplementary Tables 1,2). To assess family level-differences in both parameters, a one-way ANOVA and subsequent post-hoc Bonferroni Correction were applied (Supplementary Table 3,4). At the coral species level, gene copy numbers failed to meet the assumptions for parametric testing and were therefore subjected to a rank-based Kruskal-Wallis One-Way ANOVA and subsequent post-hoc Student-Neuwan-Keuls Multiple Pairwise Comparison. Measured gene expression met the assumptions for normality and were therefore tested with a One-Way ANOVA and subsequent post-hoc Holm-Sidak Multiple Pairwise Comparison to identify significant differences (for full details, see Supplementary Results). To test for a significant relationship between nifH gene copy number and expression rate within each of the two coral families, nonlinear regression was conducted with relative gene copy numbers as predictor and relative gene expression rates as dependent variable (Supplementary Tables 5,6).

Normalization of nifH qPCR data: 16S rRNA vs. ITS2 as a Reference Gene

nifH gene copy numbers and expression rates normalized against either 16S rRNA or ITS2 were distinctly different (Fig. 1A,B, Supplementary Figure 1). Copy numbers and expression rates after normalization against 16S rRNA did not reveal clear family level patterns,
nor aligned with nitrogen fixation activities in *P. verrucosa* and *P. granulosa*. Specifically, lowest and highest relative *nifH* gene copy numbers were detected in the Pocilloporidae (in *P. verrucosa* and in *S. pistillata*, respectively), while gene expression was highest in the fungiid coral *C. echinata*, and lowest in *P. verrucosa*. In the DOM experiments, *nifH* gene copy numbers in *P. granulosa* tissues remained low at all times, while gene expression rates were increased up to 8-fold in the soft coral exudate treatment (Supplementary Figure 1). In contrast, there was a clear family-level pattern in ITS2-referenced *nifH* gene copy numbers and expression rates, i.e. the Pocilloporidae *P. verrucosa* and *S. pistillata* exhibited ~ 4 – 14 fold increased relative gene copy numbers and expression compared to the Fungiidae, respectively (Figure 1A, Supplementary Table 2). This pattern clearly aligns with nitrogen fixation activity presented for *P. verrucosa* and *P. granulosa* in the present study and for rates previously reported for *S. pistillata* (2, 14; Supplementary Table 1). Under DOM enrichment, *nifH* gene copy numbers and expression increased significantly over 24 hours, as was expected from previous reports on the stimulative effects of DOM on diazotroph abundance and nitrogen fixation in *P. verrucosa* (Pogoreutz et al., submitted).
Supplementary Figure 1. Relative *nifH* gene copy numbers and expression rates associated with tropical scleractinian coral tissues (fold change; referenced against the 16S rRNA gene). Error bars indicate standard errors. A. Comparative species frame work: Fungiidae (*Pleuractis granulosa, Ctenactis echinata*) vs. Pocilloporidae (*Pocillopora verrucosa, Stylophora pistillata*). B.
Pleuractis granulosa under different DOM treatments. con = control; cau = Macroalgal (Caulerpa) exudate; xen = Soft coral (Xenia) exudate.
Inappropriate reference genes for qPCR normalization can distort its outcome (15). The 16S rRNA gene is appropriate to assess the coral-associated relative diazotroph abundances within single coral species under different treatments (16; Pogoreutz et al. under review). In a comparative species framework however, normalization with the 16S rRNA gene apparently distorts *nifH* qPCR results, likely due to species-specific differences in bacterial community metrics (overall abundance, community composition, evenness). Indeed, the bacterial communities of the two Pocilloporidae investigated in the present study were shown to be overwhelmingly dominated by a single bacterial OTU (17, 18). While distortion effects may not be as apparent for 16S rRNA vs. ITS2-referenced *nifH* relative gene copy numbers (Nonlinear regression, adj. $R^2 = 0.71$, $p = 0.0213$; Supplementary Table 2F), there is a clear disparity for *nifH* gene expression rates (Nonlinear regression adj. $R^2 = 0.08$, $p = 0.59$; Supplementary Figure 1A,B; Supplementary Table 2G). These findings further emphasize the discrepancy between gene copy numbers and gene expression (19), and the critical importance of expression-based approaches in functional studies on coral holobionts (9).

In contrast to bacterial abundance, *Symbiodinium* abundance in coral tissues underlies only small species-specific variations and is comparatively constant over space and time, i.e. for the majority of species, coral cells contain one single *Symbiodinium* cell (20), even though ITS2 copy numbers may vary among *Symbiodinium* lineages (12). Further, *Symbiodinium* is the major biological sink of nitrogen and incorporates the majority of microbially fixed nitrogen within the coral holobiont (21, 22). Taken together, we argue that ITS2-normalization of *nifH* gene copy numbers is a biologically meaningful predictor of diazotroph abundance in the coral tissue. While standardization with a *Symbiodinium*-specific reference gene has obvious limitations (i.e., unsuitability for endolithic diazotroph communities), we argue that it provides a more accurate normalization to address diazotrophs in a comparative coral species framework. This view is supported by the patterns of gene expression rates matching the holobiont-associated nitrogen fixation rates reported in this study.
**Supplementary Table 1.** Overview of coral species, and numbers of replicates (fragments in Pocilloporidae, individual polyps in the Fungiidae) used in the respective assays and treatments. High DOC treatments: caul = Macroalgae (*Caulerpa*) exudate treatment; xen = Soft coral (*Xenia*) exudate treatment.

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**Supplementary Table 2.** Relative tissue-associated copy numbers and expression rates of the nifH gene via qPCR and comparison of known nitrogen fixation activity in 5 common Red Sea and/or Indo-Pacific scleractinian coral taxa. qPCR data are presented relative [ddCt, or fold change] to a reference gene (the ribosomal Internal Transcriber Region 2 of *Symbiodinium*). *coral taxa not subjected to qPCR assays in the present study. con = control treatment; cau = *Caulerpa* exudate treatment; xen = *Xenia* exudate treatment; ITS2 = ribosomal Internal Transcriber Region 2; BDL = below detection limit; NA = not available.

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<th>Relative nifH gene expression rate [nifH/ITS2, fold change]</th>
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<th>Reference $N_2$ fixation</th>
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**Supplementary Table 3.** Summary of One-Way Analysis of Variance (ANOVA) and Nonlinear Regression results. A. ANOVA results for *nifH* gene expression across all coral species and treatments. B. ANOVA results for *nifH* gene copy numbers between Pocilloporidae and Fungiidae. C. ANOVA results for *nifH* gene expression between Pocilloporidae and Fungiidae. D. Nonlinear Regression on the relationship between *nifH* gene copy numbers and gene expression rates in Pocilloporidae. E. Nonlinear Regression on the relationship between *nifH* gene copy numbers and gene expression rates in Fungiidae. F. Nonlinear Regression on the relationship between *nifH* gene copy numbers normalized against 16S rRNA and ITS2-referenced across all corals and treatments. G. Nonlinear Regression on the relationship between *nifH* gene expression rates normalized against 16S rRNA and ITS2-referenced across all corals and treatments.

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### Appendix Chapter 3

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**Supplementary Table 4.** Overview of coral species, and numbers of replicates (fragments in Pocilloporidae, individual polyps in the Fungiidae) used in the respective assays and treatments. High DOC treatments: caul = Macroalgae (*Caulerpa*) exudate treatment; xen = Soft coral (*Xenia*) exudate treatment.

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<td>Gene copy number</td>
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Supplementary Table 5. Relative tissue-associated copy numbers and expression rates of the \textit{nifH} gene via qPCR and comparison of known dinitrogen (N\textsubscript{2}) fixation activity in 5 common Red Sea and/or Indo-Pacific scleractinian coral taxa. qPCR data are presented relative [ddCt, or fold change] to a reference gene (the ribosomal Internal Transcriber Region 2 of \textit{Symbiodinium}). *coral taxa not subjected to qPCR assays in the present study. con = control treatment; cau = \textit{Caulerpa} exudate treatment; xen = \textit{Xenia} exudate treatment; ITS2 = ribosomal Internal Transcriber Region 2; BDL = below detection limit; NA = not available.

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<td>480.03</td>
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<td>Shashar et al. 1994; Cardini et al. 2015</td>
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<td>\textit{P}. \textit{granulosa} (xen)</td>
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**Supplementary Table 6.** Summary of One-Way Analysis of Variance (ANOVA) and Nonlinear Regression results. **A.** ANOVA results for *nifH* gene expression across all coral species and treatments. **B.** ANOVA results for *nifH* gene copy numbers between Pocilloporidae and Fungiidae. **C.** ANOVA results for *nifH* gene expression between Pocilloporidae and Fungiidae. **D.** Nonlinear Regression on the relationship between *nifH* gene copy numbers and gene expression rates in Pocilloporidae. **E.** Nonlinear Regression on the relationship between *nifH* gene copy numbers and gene expression rates in Fungiidae. **F.** Nonlinear Regression on the relationship between *nifH* gene copy numbers normalized against 16S rRNA and ITS2-referenced across all corals and treatments. **G.** Nonlinear Regression on the relationship between *nifH* gene expression rates normalized against 16S rRNA and ITS2-referenced across all corals and treatments.

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### Appendix Chapter 3

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SUPPLEMENTARY REFERENCES


CHAPTER 4

Invariance of bacterial community composition under excess nitrogen indicates limited ability for holobiont acclimatization in the coral *Pocillopora verrucosa*

*Pogoreutz, C.*, Rädecker, N., Cárdenas, A., Gärdes, A., Wild, C., Voolstra, C. Invariance of bacterial community composition under excess nitrogen indicates limited ability for holobiont acclimatization in the coral *Pocillopora verrucosa*. This chapter is in preparation for submission to *mBio.*
ABSTRACT

The physiological performance of tropical coral holobionts is based upon complex interplays of its members: the coral host, endosymbiotic algae of the genus *Symbiodinium*, and a diverse bacterial community (among others). As *Symbiodinium* populations are maintained by the coral host via nitrogen limitation, the disruption of this limitation can induce holobiont breakdown as reflected in bleaching, disease, or mortality. Still, the functional dependencies of the holobiont on nitrogen cycling remains poorly understood. Here, we exposed the coral *Pocillopora verrucosa* to excess nitrogen conditions to understand how the holobiont response is shaped by its members. The rapid breakdown of the coral holobiont under excess nitrogen availability was manifested in the doubling of the *Symbiodinium* population and changes of its dominant clades within 14 days, and attributed to a loss of host regulative forces. Ultimately, partial host mortality (massive tissue lesioning) occurred after 13 days. Remarkably, the overall bacterial community composition remained stable even 24 h after the onset of tissue lesioning, when host mortality was observed at 14 days. Our findings suggest a strong host selection of associated bacteria in *P. verrucosa*. While this non-dynamic relationship between the bacterial community and environmental conditions in the *P. verrucosa* holobiont may be successful under moderate disturbance, it may constitute an Achilles’ heel under global environmental change.
SIGNIFICANCE STATEMENT

The widely distributed Pocillopora verrucosa coral holobiont maintains a stable bacterial community under excess nitrogen availability (up to 50-fold increased) over 14 days, even 24 h after the onset of significant partial host mortality. This strict host microbiome selectivity may constitute an advantage driving the success of P. verrucosa holobionts on unimpacted or moderately disturbed shallow-water reefs. However, this specialist strategy may be detrimental under rapidly changing environmental conditions, such as nutrient enrichment or global climate change.
INTRODUCTION

The bi-directional nutrient exchange symbiosis between the coral animal and intracellular dinoflagellate algae of the genus *Symbiodinium* is the functional basis of the adaptation of corals to nutrient-poor waters (1). Along with their algal symbionts, corals harbour a diverse microbiome, comprised of bacteria, archaea, fungi, and viruses (among others) (2). Consequently, the performance of this coral metaorganism is determined by the interactions of its individual members, and indeed, the holobiont in its entirety is considered the unit of environmental selection (3, 4).

To maintain proper holobiont functioning, the interactions among its individual members are governed by strong internal regulatory mechanisms (1, 5, 6; Pogoreutz, Rädecker et al. in prep.). Among these regulatory processes, the host-imposed restriction of nitrogen supply to *Symbiodinium* is crucial to maintain the coral-algae symbiosis (1). Specifically, this resource limitation maintains the high rates of translocation of organic carbon in the form of energy-rich photosynthates by *Symbiodinium*, which can fully meet or even exceed the coral's metabolic requirements (1). Excess nitrogen availability on the other hand rapidly releases *Symbiodinium* from nitrogen limitation, thereby destabilizing the coral-algae symbiosis. Recently, Wooldridge (7) linked 'suboptimally' high *Symbiodinium* densities *in hospite* on reefs with high bleaching sensitivity to excess seawater nutrient levels. Indeed, in order to counteract the effects of rapidly proliferating algal symbionts, corals may be able to directly regulate internal nitrogen availability via the incorporation and storage of nitrogen in their tissues (8; Pogoreutz, Rädecker et al. in prep.). Consequentially, Rädecker et al. (9) recently proposed the critical importance of microbial nitrogen cycling processes in the stabilization and destabilization of the coral-algae symbiosis, and thereby holobiont functioning.

The importance of microbial services to coral holobiont functioning is widely acknowledged (10). However, the significance of individual bacterial functional groups remains poorly understood. Coral-associated bacteria may act as a first line of defence against pathogen entry in the coral mucus (11), or are involved in metabolic processes (10) including nitrogen
(12, 13), carbon (14, 15) and sulfur cycling pathways (16, 17). Ultimately, the coral microbiome facilitates the acclimatization of coral holobionts to environmental change through rapid community restructuring ('The Coral Probiotic Hypothesis', 18) to maintain holobiont functioning and health (3, 11, 19). In general, restructuring of coral-associated bacterial communities encompasses the numerical regulation of a highly selected core microbiome coupled with changes in less abundant and more sporadic bacterial associates (20–22). However, a companion experiment (Chapter 3) provided recent evidence for a limited capacity for aclimative restructuring of the bacterial community associated with certain holobionts, for instance the common Indo-Pacific scleractinian coral *Pocillopora verrucosa*. Specifically, an absence of compositional shifts was observed in the bacterial community associated with *P. verrucosa* in a high dissolved organic carbon manipulation aimed to stimulate coral-associated nitrogen fixation activity (Pogoreutz, Rädecker et al., in prep.). In this context, our understanding particularly of the functional dependency on nitrogen cycling in corals and how it shapes the aclimative microbial restructuring in scleractinian corals remains rather limited.

By manipulatively overwhelming the host-imposed internal nitrogen limitation, we aimed to elucidate the processes underlying holobiont breakdown under excess nitrogen availability in *Pocillopora verrucosa*. This, we achieved in a 14 day manipulative *ex situ* experiment assessing physiological response and changes in the community structure of the coral holobiont.

**METHODS**

Six colonies of the brown color morph of *P. verrucosa* were collected from the Central Red Sea mid-shore reef Al-Falah (N22°18'19.98", E38°57'46.08"; Thuwal, Kingdom of Saudi Arabia). The colonies had an average diameter of 40 cm and were collected at 7 – 8 m water depth. Care was taken to sample corals at least 5 m apart from each other, a distance adequate to avoid the collection of clonal colonies of *P. verrucosa* (23). After a 4 week acclimation period at the Coastal and Marine Resources Core Lab (CMOR) at the King Abdullah University of Science
and Technology (KAUST), fragments of each of the six colonies were re-distributed among six aquarium tanks (100 L each), resulting in each tank containing on average 20 replicates of each colony. Maintenance conditions were kept constant (seawater temperature 26.91 ± 0.38 °C, salinity 40.96 ± 0.77 PSU, photosynthetic active radiation ~ 100 quanta μmol s⁻¹ m⁻², on a 12:12 h daylight cycle; dissolved oxygen levels remained > 6 mg l⁻¹ at all times). For detailed information on coral collection, fragmentation, and husbandry, see the Supplementary Information.

To overwhelm the regulation of nitrogen limitation by the coral host, we conducted a manipulative excess nitrogen (EN) treatment in three of the six tanks, while the other three tanks remained unmanipulated. At the beginning of the experiment, total (organic and inorganic) dissolved nitrogen (TDN) levels were manipulated in three of the aquarium tanks by the addition of 0.05 mg l⁻¹ of polymer-coated slow release urea fertilizer pellets (Duration Urea 45, Agrium Advanced Technologies, Inc., Loveland, CO). Urea is increasingly detected in coastal waters (24), and can be taken up by isolated Symbiodinium (25) or entire coral holobionts (26). Urea rapidly photodissociates into carbon dioxide and ammonium in aqueous solutions (27), thereby allowing a co-enrichment of dissolved organic and inorganic nitrogen. Triplicate seawater samples for TDN analyses were collected weekly.

All sampling procedures and analyses are described in detail in the Supplementary Information. Briefly, measurements and sample collection were conducted at 0, 7, and 14 days of the experiment. For physiological and molecular measurements, six and four colony replicates were used, respectively. Holobiont physiological response parameters included oxygen evolution measurements to calculate holobiont gross photosynthetic (Gp) and respiration rates (R), as well as total alkalinity measurements to calculate dark calcification rates at days 0 and 7. The maximum quantum yield was measured at all time points to assess potential photodamage to Symbiodinium. To account for changes in the Symbiodinium population (cell density, chlorophyll a content, ITS2 gene marker diversity) and bacterial community composition (based on the 16S rRNA gene marker diversity), four coral fragments (one per colony) for each
treatment and time point (total n = 4 x 2 x 3 = 24) were flash-frozen in liquid nitrogen and stored at -80 °C until further processing. Triplicate seawater samples were collected for bacterioplankton community analyses at each time point (n = 3 samples x 2 treatments x 3 time points = 18 in total) and subsequently stored at -80 °C until further processing. DNA extraction from coral tissues and filters was performed with the Qiagen DNeasy Plant kit (Hilden, Germany) according to manufacturer's instructions. Detailed information for DNA extraction, PCRs, and sequence data analysis are provided in the Supplementary Information.

RESULTS

Water Parameters and Coral Phenotype

Water parameters including nutrient concentrations in the controls remained well within the natural range for Red Sea water (Supplementary Table 1). Over time, the control tanks experienced a moderate depletion in TDN. In contrast, in the EN treatment tanks, the TDN levels increased 40 to 50 times compared to untreated seawater controls at 7 and 14 days of manipulation, respectively (Supplementary Table 1). The control coral fragments remained in a visibly healthy state. In contrast, coral fragments in the EN manipulation responded with visible tissue darkening within the first days of treatment indicating *Symbiodinum* population growth, and experienced massive tissue lesions (< 90 % tissue loss) at 13 days. A 100 % mortality was observed during the 14 day sampling, as reflected in > 90 % tissue loss. Notably, at this point, coral tissues in the EN coral fragments still remained visibly darkened. The remaining tissues were collected, flash-frozen in liquid nitrogen, and stored at -80 °C until further processing for *Symbiodinum* and bacterial community analysis. Light microscopy of lesioned coral tissue samples from EN day 14 fragments revealed an abundance of large free-living ciliates containing ingested *Symbiodinum* cells (Supplementary Figure 1).
Highly significant treatment responses were found for *Symbiodinium* density, chlorophyll a content, and dark calcification (Figure 1). The *Symbiodinium* population responded with a rapid increase in density by 79 and 108 % relative to controls at days 7 and 14, respectively. Simultaneously, relative cell chlorophyll a contents increased by 30 and 20 %, respectively (Figure 1). Concomitantly, dark calcification rates were significantly decreased by 77 % in the EN treatment after 7 days (Generalized Estimating Equations General Linear Models or geeglm; see Supplementary Table 2 for full model statistics). Despite these drastic effects on *Symbiodinium* populations, no significant treatment effects were observed for gross photosynthesis (P₀), the maximum quantum yield, or holobiont respiration rates (R) when normalized to coral surface area (Supplementary Table 2 for full model statistics; Supplementary Figure 2). Normalized to *Symbiodinium* cell density, however, this equals an overall decrease in Gₚ performance by 44 % compared to day 0 after 7 days.

**Figure 1.** Physiological responses of the *Pocillopora verrucosa* holobiont in control and excess nitrogen treatments over time. A. Cell density of *Symbiodinium* in the tissues of *P. verrucosa*. B. *Symbiodinium* cell chlorophyll a [relative fold change to control]. C. Holobiont-associated dark calcification rates. N = nitrogen. Asterisks denote statistically significant differences (p < 0.05) as apparent from (gee)glms and Bonferroni post-hoc corrections, if applicable. 0, 7, 14 = days from the start of the experiment.
Symbiodinium community changes

A total of 24 ITS2 gene libraries with a total of 2,224,452 reads with an average length of 283 bp (distributed over 4 coral replicates x 2 treatments x 3 time points = 24) were generated. The Symbiodinium communities were dominated by the clades A1 and D6 in varying relative abundances, cumulatively accounting for 98 – 100 % of the total Symbiodinium reads (Figure 2A). The remaining sequences included Symbiodinium clades D7 and C15, cumulatively comprising 0 – 2 % of all sequences.

Of the total sequence counts across all fragments, A1 and D6 cumulatively contributed on average 50 – 95 % and 1 – 48 %, respectively. There were significant overall effects on ITS2 composition across all time points and treatments, a significant effect of time in the EN treatment, and significant treatment effects at day 14 (amovas, for full model details, see Supplementary Table S3). Specifically, at 14 days, clade D6 contributed on average with 35 % to Symbiodinium populations in the control treatment, while this clade comprised only 1 % of all reads in the EN treatment (Figure 3A).

Bacterial Community Changes

For the bacterioplankton and coral tissue-associated bacterial communities, we generated a total of 42 16S rRNA gene libraries, comprising a total of 5,955,453 sequences, respectively (distributed over 4 coral replicates x 2 treatments x 3 time points = 24, plus 3 seawater sample replicates x 2 x 3 = 18; resulting in total n = 42). After removing chloroplast, mitochondria, archaea, eukaryotic and chimeric sequences and unknown reads, a total of 1,761,968 reads with an average length of 309 bp were retained for further analyses. Classification against the 2015 version of the Greengenes database (28, 29) yielded 2,236 distinct OTUs for the bacterioplankton and the coral-associated bacterial communities (comprised by 1502 and 1228 distinct OTUs, respectively).

The coral-associated bacterial community significantly differed from the bacterioplankton community (amova, p < 0.001; for full details, see Supplementary Table 4).
the remainder of the analyses, we focused on coral-associated bacterial communities, which were dominated by Gammaproteobacteria (57 % of total reads), Actinobacteria (20 %), and Chlamydia (13 %). The overall community composition did not exhibit significant differences between the control and EN treatments at any time point (Figure 3B; amova, Supplementary Table 3; for full details, see Supplementary Table 4). Over all samples, the community was dominated by bacteria of the family Endozoicomonaceae (∼30 – 80 %; order Oceanospirillales), followed by Brevibacteraceae (5 – 30 %; order Actinomycetales) and Simkaniaceae (3 – 45 %; order Chlamydiales) (Figure 3B). The core microbiome, i.e. the OTUs consistently shared with all coral samples, consisted of unclassified Endozoicomonaceae (OTU0001, OTU0005; 41 and 17 %, respectively, and cumulatively 58 % of all sequences) and Brevibacteraceae (OTU0003; 15 % of all sequences over all samples). In total, the members of the core microbiome accounted for 73 % of the total sequences over all samples (for a complete presentation of coral-associated OTU abundances, see Supplementary Table 5). There was no change in bacterial alpha diversity indices over time and due to treatments (Supplementary Table 4, anova, p > 0.05).

Functional profiling of the bacterial community based on phylogenetic inference via METAGENassist (30) showed that the P. verrucosa-associated bacterial community covered a range of nutrient cycling pathways, specifically nitrogen (nitrogen fixation, nitrite reduction, ammonia oxidation) and sulphur cycling (sulfate reduction, sulfide oxidation). Further, there was an association with metabolic processes involving the breakdown of (complex) organic molecules (xylan, chitin, chlorophenol degraders, sugar fermenters), as well as polyhydroxybutyrate (PHB) storage.

While initially only 1 bacterial indicator OTU was identified for all coral fragments at day 0, 40 indicator (i.e. significantly associated) OTUs emerged in EN fragments at 14 days (for detailed information, see Supplementary Table 6). Even cumulatively, these indicators made a minor contribution to the overall bacterial community in EN coral fragments at day 14, and therefore did not affect the overall community composition. Specifically, indicators comprised 0.15 % of the total EN 14 day coral sequences, the most abundant indicator OTU0002, the
Rhodobacter *Nautila*, comprising 0.08% of the total. The majority of bacterial indicators yielded GenBank matches with uncultured bacteria, 25 of which were classified at the family level (n = 5 each: Rhodobacteraceae, Saprospiraceae; n = 3 each: Alteromonadaceae; n = 2 each: Bacteriovoracaeae, Bdellovibrionaceae. Remaining families with n = 1 each are presented in the Supplementary Table 5). Seven indicator OTUs were previously associated with tropical corals or reef environments, 5 of which in association with disease, including *Vibrio shilonii*. A number of OTUs were previously associated with aquatic sediments, biofilms, or biofouling (for detailed information, see Supplementary Table 5).
Figure 2. Coral tissue-associated bacterial community composition in *Pocillopora verrucosa* in control and excess nitrogen treatments over time. A. Mean proportions of the dominant *Symbiodinium* (n = 4 each), in relation to changes in population density. The overall community exhibited significant compositional fluctuations in its two clades A1 and D6 (amova, Fs = 193.259, p = 0.04). The relative contribution of the two clades was significantly different at 14 days (amova, Fs = 650.314, p = 0.05). B. Mean proportions of the 6 most abundant bacterial OTUs (n = 4 each) annotated on class (inner circles circles) and OTU levels (outer circles). The overall bacterial communities remained stable throughout the entire experiment regardless of treatment (amova, F = 1.332, p = 0.144).
DISCUSSION

To understand the potential for acclimatization in *P. verrucosa*, we investigated the responses of the holobiont, *Symbiodinium*, and bacterial communities under EN conditions. Specifically, the *Symbiodinium* population exhibited marked changes in population density and composition within 14 days only, which were linked with significantly decreased coral calcification, suggesting a strong functional dependency on nitrogen availability. In contrast, the overall tissue-associated bacterial community remained stable over time and regardless of treatments. This finding suggests that the main driver of the bacterial community structure is host selective forcing rather than nitrogen dependence in *P. verrucosa*. Host regulative processes only appeared to be disengaged 24 hours after the onset of partial mortality under EN conditions at 14 days, when the emergence of new significant (albeit very rare) bacterial associates was detected, but no significant treatment effects in the overall community composition. Here we discuss the implications of our findings in the context of acclimatization and adaptation potential of *P. verrucosa* under rapid environmental change.

Breakdown of the Coral-Algae Symbiosis

The EN treatment rapidly disrupted the coral-algae symbiosis by releasing *Symbiodinium* from nitrogen limitation (1, 31), thereby impressively underlining the strong host regulation of algal symbiont populations by the host (32). The overcoming of host-regulated nitrogen limitation resulted in a doubling of *Symbiodinium* population density coupled with increased relative chlorophyll *a* content per cell, an absence of photophysiological changes and a decrease (albeit non-significant) in holobiont respiration. While no statistically significant treatment effects on the holobiont level were apparent for P*o*, normalization to individual *Symbiodinium* cells revealed that the algal symbiont communities in the EN treatment performed at about half the rates of the control fragments. In combination with increased chlorophyll *a*, this drop could likely be attributed to the effects of self-shading or carbon dioxide limitation at higher population densities or due to attenuation effects in algal cells with elevated chlorophyll *a* content (33, 34), and/or to nutrient-imbalanced growth in *Symbiodinium* (35). The latter may
have shifted the algal symbionts from nitrogen limitation to phosphorus starvation, which supposedly can result in the improper functioning of the photosynthetic apparatus in *Symbiodinium*, and subsequently increased susceptibility to coral bleaching (36). While increased *Symbiodinium* densities *in hospite* are considered a common trophic response to elevated nutrient availability *in situ* (37, 38), Wooldridge (2016) recently linked bleaching susceptibility in corals with 'suboptimally' high *Symbiodinium* densities (∼1 – 3 x 10⁶ cells cm⁻²; species-specific). However, even though no bleaching was observed in the present study due to the absence of heat and light stress, holobiont breakdown in the form of tissue lesions occurred at 13 days, and mortality on day 14. Similar to reports on bleached coral holobionts however (39), we observed reduced respiration rates associated with EN corals in the current study. It was proposed earlier that reduced photosynthetic performance of *Symbiodinium* would likely result in lower rates of inorganic carbon (CO₂) acquisition and fixation, and, subsequently, decreased rates of fixed carbon translocation to the host by *Symbiodinium* (39). Reduced translocation of photosynthates would therefore ultimately result in lower holobiont respiration due to reduced energy availability for coral metabolism (39).

Coral calcification exhibited a steep decline in the EN treatment within only 7 days. This suggests that EN availability released *Symbiodinium* from nitrogen limitation, relieving the algal symbionts from the requirement to translocate most of their photosynthates to the coral host. Ultimately, increased amounts of organic carbon are allocated towards the algal symbiont’s own metabolism, thereby depriving the coral host of its nutrition (40; Pogoreutz, Rädecker et al. in prep.). Additionally, high inorganic nitrogen availability may have already affected the rates of carbon acquisition and fixation by *Symbiodinium*, resulting in lower overall carbon translocation rates (41).
Changes in Symbiodinium Communities

The rapid doubling of the *Symbiodinium* population *in hospite* under EN conditions illustrates the strict host-regulated nitrogen limitation in nutrient-poor reef waters (1, 42). In the present study, this rapid proliferation of algal symbionts was associated with significant relative compositional fluctuations of the two most dominant symbiont clades, A1 and D6. The latter almost entirely disappeared under EN within 14 days. In general, Red Sea *P. verrucosa* exhibit a rather stable association with *Symbiodinium* A1, except in the northern- and southernmost regions. In contrast, D6 was previously reported only from background abundances in this Red Sea holobiont (43, 44).

Differential adaptive physiological mechanisms among different *Symbiodinium* clades, for instance differential inorganic carbon (45) or nitrogen acquisition capability (8, 46), photosynthetic characteristics (47, 48), and photorepair mechanisms (49), affect holobiont performance under stress (45). In the present study, we observed the rapid disappearance of D6 to background levels in the high nitrogen regime. Previously, clade D *Symbiodinium* have been reported to exhibit an inferior capacity for nitrogen acquisition compared to other clades at ambient temperatures (50, 51). The rapid outcompetition of *Symbiodinium* clade D6 by clade A1 in the prevailing EN regime thus suggests a high nitrogen acquisition efficiency in the latter.

The efficient nitrogen uptake in *Symbiodinium* A1 may explain its prevalence in *P. verrucosa* along almost the entire Red Sea basin (52). Whilst *Symbiodinium* clade A associations are rare in most scleractinian corals (53), they often occur in health-compromised holobionts (54). Clade A is considered opportunistic, as it is associated with low fixed carbon translocation rates to the host (54). Our results suggest that this reduced carbon translocation could be consequential of a highly efficient nitrogen uptake in *Symbiodinium* from clade A. Taking the highly oligotrophic conditions of the Red Sea into account however, the association with clade A1 *Symbiodinium* may be strongly selected for, as it may increase holobiont fitness by sustaining high holobiont productivity. In contrast, EN scenarios may reduce holobiont fitness via host starvation (Pogoreutz, Rädecker et al. in prep.) in clade A1-associations.
**Bacterial Community Changes**

The overall coral-associated bacterial community remained structurally and functionally stable, despite the EN treatment. This finding contrasts with the Coral Probiotic Hypothesis and previous reports on distinct bacterial community changes under high nutrient enrichment (18, 55) or other environmental change (20, 21, 56). Interestingly, a significant amount of rare bacterial associates emerged in the EN treatment after 14 days, a substantial number of which belonging to opportunistic and potentially pathogenic groups (for a detailed overview, see the Supplementary Discussion), corroborating our view of the overall breakdown of host regulation. Specifically of note is the emergence of the putative pathogens *Vibrio shilonii* (57) and *Nautilia* (58). Together with the occurrence of *Symbiodinium*-devouring ciliates previously observed in diseased corals, this can likely be attributed to the overwhelming of host defence mechanisms and subsequent pathogen entry (59, 60).

**Implications for Acclimatization and Adaptation Potential in *P. verrucosa***

The Coral Probiotic Hypothesis postulates rapid acclimative responses of the coral-associated microbiome via community restructuring within days to weeks (18). While recent studies have indeed reported on bacterial restructuring in corals under chronic exposure to changes in water quality or salinity (Jessen et al. 2013; Ziegler et al. 2015; within 28 days in *Fungia granulosa* exposed to high salinity treatments: Röthig et al. 2016), no shift in the overall community composition due to treatment effects was observed in *P. verrucosa* in a companion experiment featuring high dissolved organic carbon additions (Pogoreutz, Rädecker et al., in prep.) and in the current study, even 24 hours after the onset of massive tissue lesions under EN conditions. In contrast, the *Symbiodinium* community exhibited distinct compositional fluctuations. Considering the rapid population increase however, these fluctuations were likely not acclimative or selective, but rather a consequence of the host losing control over its algal symbionts. The maintenance of a stable tissue-associated bacterial community however has interesting implications for the evolutionary biology of *P. verrucosa*. Different to the mechanisms
brought forth by the Coral Probiotic Hypothesis, *P. verrucosa* may strongly select for highly structured and identical rather than rapidly responding bacterial communities. Latitudinal and water depth patterns in physiology as well as the stable *P. verrucosa-Symbiodinium* clade A1 association along the Red Sea basin suggest a broad ecological niche and low local adaptation in this holobiont (43, 44, 52). Together with a stable and highly structured bacterial community, this tripartite association may form a physiological ‘generalist prototype’ with a broad ecological niche, yet low competitiveness. While this prototype may perform best in a small geographic range only, it appears to be sufficiently successful to prevail along the entire Red Sea basin (52).

Highly structured and selected microbiomes may constitute an evolutionary advantage under stable conditions. Indeed, shifts towards fast-growing and weedy Pocilloporidae on impacted reefs suggest a strong selection for this strategy under disturbance (61, 62). Interestingly, this stable tripartition may also reduce the risk of disease, as reflected in regionally low disease occurrence and prevalence in *Pocillopora* corals (63). In combination with the stability of Red Sea *P. verrucosa-Symbiodinium* clade A1 association and evidently low regional physiological adaptation, this suggests high dispersal and gene flow (52). Indeed, absence of genetic differentiation among *P. verrucosa* populations along some 850 km of the Red Sea coast were shown recently (23). Similarly, stable and highly specific *Pocillopora-Symbiodinium* associations (albeit not necessarily with clade A; 64) in combination with low genetic differentiation over large spatial scales have been reported from Indo-Pacific *Pocillopora* populations (64), further supporting holobiont selection by the host. To add to this interesting emerging picture, it was recently reported that the pocilloporid coral *P. verrucosa* hosts the same genotype of the bacterium *Endozoicomonas* across seven major geographic regions (66). This pattern of symbiont selection was attributed to the reproductive strategy of *P. verrucosa*, which is broadcast spawning (66). [In contrast, the pocilloporid *Stylophora pistillata* hosts geographically distinct *Endozoicomonas* phylotypes, which can likely be related to its list history as a brooder (66)].
The absence of bacterial community restructuring under EN conditions in combination with the emergence of rare opportunistic indicators only after the onset of partial mortality may be attributed to the inherent association of Red Sea P. verrucosa with its core bacterial microbiome. Strong selective forcing by the host likely maintains the core microbiome under changing environmental conditions, suggesting an obligate symbiotic relationship with one or several of its associates. Endozoicomonaceae are abundant in scleractinian corals from different families, including Acroporidae (22), Poritidae (67), and Fungiidae (68), but overwhelmingly dominate the bacterial communities of Pocilloporidae, for instance P. verrucosa (22, 66), P. damicornis, S. pistillata (66, 69), and Seriatopora histrix (70). This pattern conserved on the family level supports the potentially critical importance of the Pocilloporidae-Endozoicomonas association for holobiont functioning, as previously suggested (71, 72). Specifically, Endozoicomonaceae negatively correlate with the occurrence of bleaching and associated pathogens, suggesting a critical role in photoprotective mechanisms and co-evolution with the coral host and Symbiodinium (70).

Highly selectived holobionts however may be prone to distinct limitations. Processes underlying the selection of bacterial symbionts are likely related to high maintenance costs for the sensory and regulatory machinery involved. Lag-time limits on the other hand may constrain fast acclimatization responses, thereby ultimately reducing the holobiont’s overall fitness under rapidly changing environmental conditions (73). Despite their Indo-Pacific wide success and their ability to cope with a wide range of temperature and nutrient regimes (52), Pocilloporidae are highly susceptible to environmental stress, including turbidity (44) and ocean warming (74). These corals may not be able to overcome the trade-offs associated with the selected holobiont strategy in a changing world. Indeed, global environmental change may not select for such holobionts in the long term, but rather favour holobionts with a high capacity for flexible microbiomes.
ACKNOWLEDGEMENTS

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REFERENCES


APPENDIX

Chapter 4

Supplementary Information
Supplementary Information Chapter 4:

Invariance of bacterial community composition under excess nitrogen indicates limited ability to holobiont adjustment in the coral *Pocillopora verrucosa*

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Co-correspondence: Christian Wild, Christian R. Voolstra

**Supplementary Information**
SUPPLEMENTARY METHODS

Coral Collection

Six colonies of the brown color morph of *P. verrucosa* were collected from a water depth of 8 – 10 m off the northern tip of al-Fahal Reef, Central Red Sea, off Thuwal, Saudi Arabia (N22°18’19.98”, E38°57’46.08”). Al-Fahal Reef is not subject to any legislative protection or special designation as a marine/environmental protected area. The Saudi Coastguard Authority under the auspices of KAUST University issued sailing permits to the site that includes coral collection. The coral *P. verrucosa* is listed as least concern on the IUCN Red List (http://www.iucnredlist.org/details/133197/0).

Experimental Setup and Sampling

Experiments were conducted at the wet lab facility of the Coastal and Marine Resources Core Lab (CMOR) at the King Abdullah University of Science and Technology (KAUST, KSA). The aquarium system consisted of two identical units, consisting of three closed replicate experimental tanks (100 L) connected to reservoir bins (100 l each) containing heating equipment. Natural Red Sea reef water was circulated in each tank and thirty percent of the water was replaced on a daily basis, maintaining close to natural water parameters. Maintenance conditions were kept constant (temperature 26.91 ± 0.38 °C, salinity 40.96 ± 0.77 PSU, photosynthetic active radiation ~ 100 quanta μmol s⁻¹ m⁻², on a 12:12 h daylight cycle; dissolved oxygen levels remained > 6 mg l⁻¹ at all times). Individual colonies were fragmented, glued to 40x40 mm stone tiles with a two-part epoxy putty (Reef Construct, AQUA MEDIC GmbH, Bissendorf, Germany), and acclimated for a period of 4 weeks in six aquaria. Fragments were then distributed among the aquaria, resulting in each aquarium containing replicates of each colony.

At the start of the experiment, total nitrogen dissolved levels (TDN) were manipulated in three of the experimental tanks by addition of 0.05 mg l⁻¹ of polymer-coated slow-release urea fertilizer pellets (Duration Urea 45, Agrium Advanced Technologies, Inc., Loveland, CO).
Diffusion bags containing the pellets were deployed in the reservoir bins at the beginning of the experiment, and replenished after 1 week. The other three aquaria were maintained at ambient sea water nutrient levels. Urea was selected for the excess nitrogen (EN) manipulation as it constitutes the most widely applied nitrogenous fertilizer, is used in industrial and agricultural operations, and introduced via anthropogenic terrestrial run-off into sensitive coastal waters, where it is detectable in increasing concentrations (23, 24). It is readily taken up by a variety of marine organisms, including bacterio- and phytoplankton (23, 25), but also coral holobionts (26). Urea is not environmentally inert, but is rapidly hydrolyzed into ammonium and carbon dioxide via photodissociation (27) and potentially experiences further degradation downstream (i.e., nitrates) (23), thereby permitting a co-enrichment of dissolved organic and inorganic nitrogen species.

For physiological and microbial analyses, replicates (fragments) from six and four colonies were selected, respectively. Measurements of the maximum quantum yield of photosystem II (PS) of *Symbiodinium in hospite* were conducted and samples for microbial community parameters (*Symbiodinium*, coral- and seawater-associated bacterial communities) collected at day 0, 7, and 14 of the manipulation. Rate function parameters were measured or sampled on day 0 and 7 only. For invasive response parameters, at each time point single fragments originating from all mother colonies and treatments were rinsed with filter-sterilized seawater (FSW; 0.22 μm), flash-frozen in liquid nitrogen and stored at -80°C until further processing. Seawater samples for bacterioplankton community analyses were collected in triplicates (1 l each) for each treatment and time point. The Seawater samples were filtered through 0.22 μm, and the filters immediately frozen and stored at -80°C until further processing.

**Nutrient Measurements**

Treatment water samples for the analysis of total dissolved nitrogen (TDN) at the micromolar level were collected at all sampling points in 30 ml triplicates. Each water sample was filtered (0.7 μm glass fiber filters, Fisher Scientific) and frozen at -20°C. Frozen water
samples were sent to the Marine Chemistry Lab (University of Washington, Seattle) for analysis according to standard method (SM) 4500-P J (28).

**Physiological Measurements**

Gross photosynthetic (PG) and dark respiration (R; calculated via O₂ evolution), as well as dark calcification rates were quantified during incubations in gas-tight 1 L glass chambers. Six fragments per treatment were assayed each, resulting in a total number of 12 fragments subjected to a repeated measures design (i.e., the same fragments were measured during the two time intervals). Four additional chambers per treatment filled with the respective treatment water (i.e., control and TDN manipulated seawater) served as controls for planktonic background metabolism. During incubations, chambers were submersed in a water bath to facilitate maintenance of constant temperature, and constantly stirred (600 rpm). All rates were corrected for the respective mean seawater control signals and normalized to incubation time and coral surface area, which was quantified by wax coating (29).

Oxygen evolution was based on the difference of O₂ concentrations at the start and end of the same incubations during light and dark (<2 h each to avoid supersaturation of O₂), respectively, with a salinity-corrected optode sensor (FDO®925 – Optical Dissolved Oxygen Sensor, MultiLine® IDS 3440, WTW GmbH, Weilheim, Germany). Light and dark calcification rates were determined by conversion of changes in total alkalinity of filtered (0.45 μm, Nalgene® polyethersulfone membrane syringe filter) 50 ml water samples by the alkalinity anomaly technique (30).

Photosynthetic activity of *Symbiodinium* cells *in hospite* was confirmed by measuring PSII maximum quantum yield (Fᵥ/Fₘ) of dark-adapted coral fragments (12 per treatment) 1 h into the 12 h dark phase. Measurements were carried out using a pulse amplitude modulation fluorometer (DIVING-PAM, Walz, Germany).

*Symbiodinium* cells were freshly isolated from coral tissue by NaOH extraction (31). Subsamples of individual coral fragments were incubated in 1M NaOH. After 1 h, the skeleton
was removed. Suspended *Symbiodinium* cells were spun down in a bench-top centrifuge for 5 min at 3,000 RCF, the supernatant discarded, and the *Symbiodinium* pellet resuspended in 1 ml PBS (1x). After a second centrifugation step, the pellet was resuspended in a 10 % PBS-buffered formaldehyde solution and stored at 4°C until further processing. *Symbiodinium* density and relative chlorophyll a content were determined using flow cytometry (BD LSRFortessa, BD Biosciences, USA).

**Symbiodinium and Bacterial Communities**

Before DNA extraction, each frozen coral fragment was transferred into a sterile zip-lock bag. While thawing, fragments were doused with 5 mL Qiagen AP-1 tissue lysis buffer (Qiagen Plant Mini Kit, Hilden, Germany) and subsequently air-blasted using airflow from a sterile pipette tip (1000 μL filter barrier tips, Neptune, USA) on ice. Care was taken to exclude any skeletal fragments. The coral tissue slurry in AP-1 was transferred into 2 ml Eppendorf tubes and stored frozen at -80°C.

DNA was extracted from the tissue slurry and seawater filters using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. 200 μl tissue slurry in 200 μl AP-1, 4 μl RNase A stock solution (100 g/ml), and 200 μl 0.5 mm sterile glass beads (BioSpec, Bartlesville, OK) were bead-beaten at 30 Hz for 90 s with a Tissue Lyser II (Qiagen, Hilden, Germany). The bead-beat step was included to disrupt *Symbiodinium* cells. Extracted DNA was quantified and quality checked using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

PCR amplifications were performed in triplicate reactions with Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) All primers contained Illumina adapter overhangs (underlined below; Illumina, San Diego, CA, USA). For *Symbiodinium* typing, we amplified the ribosomal Internal Transcribed Spacer 2 (ITS2) region, a multi-copy genetic marker commonly used to assess *Symbiodinium* diversity (12). For amplicon-specific PCRs, we used the primers ITSintfor2 5’- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3’ and Miseq-ITS2-
reverse

5'-

GTCTCGTGGGCTGGGATCATATGCTTAAGTTGACGCGGT-3'

were used at the following PCR conditions: 94°C for 15 min, then 35 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 30 s, followed by one cycle of 72°C for 10 min and 4°C hold (12). To amplify the eubacterial 16S rRNA gene, we used the primers 16SMiSeqF-Andersson 5'-TCG TCG GCA GCGTCA GAT GTG TAT AAG AGA CAG AGG ATT AGA TAC CCT GGT A-3’ and 16SMiSeqR-Andersson 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCR RCA CGA GCT GAC GAC-3’ that target the variable regions 5 and 6 of the 16S rRNA gene (32) and amplify well with DNA extracted from coral (17, 33). For individual PCR reactions, DNA was aliquoted to 12 - 50 ng/μL, with 10 μL Qiagen Mix, 0.5 μL of each 10 μM primer mix, 1 μL of DNA template, and RNase-free water to adjust the reaction volume to 20 μl. The thermal conditions for ITS2 and 16S rRNA PCRs were as follows (respectively): 94°C for 15 min, then 35 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 30 s, followed by one cycle of 72°C for 10 min and hold at 4°C; and 95°C for 15 min, followed by 27 cycles of 95°C for 40 s, 55°C for 40 s, 72°C for 40 s, and a final extension cycle of 72°C at 10 min. 10 μl of each PCR product was run on an 1% agarose gel to visualize successful amplification. Sample triplicates were subsequently pooled and then purified using the Agencourt AMPure XP magnetic bead system (Beckman Coulter, Brea, CA, USA). Purified PCR products were subjected to an indexing PCR to add Nextera XT indexing and sequencing adapters (Illumina) according to the manufacturer’s protocol. Indexed products were again purified, quantified on the BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and QuBit (Quant-IT dsDNA Broad Range Assay Kit; Invitrogen, Carlsbad, CA, USA), and pooled in equimolar ratios. The final pooled library was purified on a 2% agarose gel to remove excess primer dimer. The library was sequenced at 8pM with 10% phiX on the Illumina Miseq, 2x 300 bp end version 3 chemistry according to the manufacturer's specifications at the Bioscience Core lab (KAUST, Saudi Arabia).
**Statistical Procedures**

Physiological parameters

For statistical analysis of response parameters, univariate linear models were fitted to our data in R v3.3.0 (34). \( P_G, P_N, R \) and calcification 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 (35). Similarly, *Symbiodinium* density, and seawater nutrient concentrations were tested with 2-factorial generalized linear models (GLMs). 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 Bonferroni post-hoc corrections. All data are reported as mean ± SE, asterisks indicate statistically significant differences (* \( p < 0.05 \), ** \( p < 0.01 \)).

Microbial community analysis

For amplicon analysis of coral tissue-associated eubacterial communities, *mothur* v1.36.1 was used (36). Briefly, reads were demultiplexed, sequences were quality trimmed, and pre-clustered (2 bp difference; 54). Sequence reads were split according to barcodes, assembled to contigs, and quality trimmed. Identical sequences (duplicates) were merged using the *unique.seqs* command, and *count.seqs* was used to calculate the number of sequences over samples represented by the remaining representative sequence. Next, singletons and rare sequences (\( n < 10 \) over all samples) were removed. Remaining sequences aligned against the SILVA database (release 119; 55) and pre-clustered (37). Chimeric sequences were removed using the UCHIME command (39) and reads assigned to chloroplasts, mitochondria, archaea, and eukaryotes were excluded. Sequences were classified into operational taxonomic units (OTUs) against Greengenes database (release gg_13_8_99; bootstrap= 60; 97 % similarity cut-off chosen to obtain OTUs; McDonald *et al.*, 2012). Bacterial community composition pie charts were created on the OTU and class level using the means of relative abundances from replicates.
(n = 4). Changes in the ‘core’ bacterial OTUs were assessed descriptively for the time points and treatments in *mothur* (command: `get.coremicrobiome`). Indicator species analysis to identify any OTUs significantly associated with time points (days 0, 7, 14) and treatments (control, EN) were conducted in *mothur* (command: `indicator`). The representative sequence of each indicator OTU was then BLASTed against the NCBI GenBank database to identify previous occurrences of identical or genetically close matches. Alpha diversity measures (number of OTUs, Chao estimate of species richness, inverse Simpson index, Simpson evenness) and an analysis of molecular variance (amova) were subsequently calculated as implemented in *mothur*.

To delve into potential functional profiles based on 16S diversity, we used METAGENASSIST for automated taxonomic-to-phenotypic mapping (41). Input files were created based on all samples in *mothur* (`make.shared` and `classify.otu` commands). During data processing in METAGENASSIST, all distinct OTUs were assigned, mapped, and condensed into 292 functional taxa and filtered based on interquartile range (42). After filtering, 69 functional taxa remained and were normalized over samples by sum and over taxa by range scaling. The data were next analyzed for ‘metabolism by phenotype’ with the Pearson distance measure and Ward clustering algorithm to visualize the results in a heatmap.
SUPPLEMENTARY RESULTS

Supplementary Table 1. Maintenance conditions in experimental seawater tanks over time. Values are presented as mean ± SE. C = control; EN = excess nitrogen treatment; TDN = total dissolved nitrogen content.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Temperature [T°C]</th>
<th>Salinity [PSU]</th>
<th>TDN [μM]</th>
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<tr>
<td>0</td>
<td>C</td>
<td>25.9 ± 0.11</td>
<td>40.8 ± 0.09</td>
<td>16.76 ± 2.67</td>
</tr>
<tr>
<td></td>
<td>EN</td>
<td>26.1 ± 0.09</td>
<td>40.9 ± 0.11</td>
<td>16.49 ± 0.35</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>26.5 ± 0.15</td>
<td>40.9 ± 0.11</td>
<td>12.95 ± 0.83</td>
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<td></td>
<td>EN</td>
<td>26.8 ± 0.12</td>
<td>40.6 ± 0.15</td>
<td>589.77 ± 1.57</td>
</tr>
<tr>
<td>14</td>
<td>C</td>
<td>26.5 ± 0.16</td>
<td>40.5 ± 0.18</td>
<td>10.68 ± 0.05</td>
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<tr>
<td></td>
<td>EN</td>
<td>26.9 ± 0.11</td>
<td>40.5 ± 0.15</td>
<td>793.09 ± 3.56</td>
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Supplementary Table 2. Model results of geeglm (**) and glm (*) for individual response parameters.

<table>
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<tr>
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<th>$X^2$</th>
<th>Df</th>
<th>n</th>
<th>P</th>
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<td>24.00</td>
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<tr>
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<tr>
<td>Gross photosynthesis*</td>
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<tr>
<td>treatment</td>
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<td>0.03</td>
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<tr>
<td>treatment x time</td>
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<td>Respiration*</td>
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</tr>
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**Supplementary Table 3.** Results of analysis of molecular variance (amova) of overall *Symbiodinium* ITS2 and bacterial 16S rRNA sequences over all sampling points and treatments and comparisons between individual time points and treatments. Significant differences are presented in bold italic. C = control treatment; EN = excess nitrogen treatment. 0, 7, 14 = duration under exposure to treatment (in days)

<table>
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<th>Group</th>
<th>Among</th>
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<th>Total</th>
<th>Fs</th>
<th>p</th>
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**Bacterial Community Composition**

- seawater vs. C7-EN7

162
## Appendix Chapter 4

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Supplementary Table 4. Summary of alpha diversity indices (means ± SE) of bacterial communities associated with the *Pocillopora verrucosa* holobiont in two treatments over time. The coverage was > 99.997 in all samples. C = control treatment. EN = excess nitrogen treatment. OTU = operational taxonomic unit.

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**Supplementary Table 5.** OTU abundance table for tissue-associated bacterial communities in *Pocillopora verrucosa* subjected to control and EN treatments (bootstrap value given only if <100). C = control; EN = excess nitrogen; Rn = replicates. 0, 7, 14 = duration of exposure (in days) of treatments.*

*Note: this is a shortened (only 25 most abundant coral-associated bacterial OTUs displayed) and simplified version of the original table to accommodate the requirements for printing and online formatting of this thesis. The original version of this table will be published as an excel sheet containing all bacterial OTUs and the full taxonomic annotation and representative sequence in the online Supplementary Information of this publication.

<p>| OTU1 Endozoicomonaceae, uncultured | 129 | 21083 | 11604 | 35104 | 11272 | 19824 | 16936 | 28062 | 14430 | 17373 | 13559 | 24949 | 189398 | 172530 |
| OTU3 Brevibacterium casei | 9274 | 13776 | 3652 | 12853 | 10512 | 4764 | 15638 | 123 | 3902 | 6234 | 5266 | 6249 | 1768 | 2911 | 3467 | 6703 | 30803 |
| OTU14 Brachybacterium, uncultured | 2123 | 1645 | 6184 | 1561 | 6381 | 5504 | 12991 | 9770 | 14062 | 1774 | 5736 | 8253 | 148531 | 21561 |
| OTU17 Denitrovibrio, uncultured | 68 | 2 | 263 | 15 | 12 | 3 | 1 | 110 | 59 | 3 | 129 | 63 | 11 | 23 | 78 | 1803 |
| OTU20 Photobacterium, uncultured | 12216 | 2 | 2 | 20 | 193 | 237 | 329 | 727 | 80 | 141 | 208 | 470 | 12425 | 2554 | 2319 |
| OTU21 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 173 | 7 | 61 | 63 | 13523 | 12048 | 20914 |
| OTU22 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 4740 | 14704 | 12048 | 20914 |
| OTU23 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU24 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU25 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU26 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU27 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU28 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU29 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU30 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |
| OTU31 Rhodobacter, uncultured | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13523 | 20914 | 20914 |</p>
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*OTU54_Arcobacter, unclassified*

*OTU53_Aquificales, unclassified*

*OTU56_Annelida*

*OTU59_Arthropoda*

*OTU62_Deltaproteobacteria, unclassified*

*OTU65_Fusibacter, unclassified*
**Supplementary Table 6.** Summary of significant s/bacterial taxa associated with coral tissue-associated bacterial communities from different time points (0, 7, 14 days) and treatments, based on indicator species analysis (significance threshold (p < 0.05). Bold nearest relatives indicate coral or reef-association. C = control; EN = excess nitrogen coral fragments; OTU = Operational Taxonomic Unit.

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[QG274054.1] uncultured marine bacterium [JN119138.1] | unpublished

[RU632811.1] uncultured bacterium. red algae [KU637194.1] | unpublished

[LCl38656.1] uncultured bacterium. deep-sea ferromanganese crusts | unpublished


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[FJ656484.1] uncultured bacterium. marine sediments | Park et al. 2010

[KP266384.1] uncultured bacterium. lacustrine sediments | unpublished

[FJ203546.1] uncultured bacterium. white-plague diseased coral *Montastrea faveolata* [FJ403095.1] | unpublished


[KU627169.1] uncultured bacterium. red algae | unpublished

[FJ391710.1] uncultured bacterium. hypoxic marine sediments | unpublished


[HE574893.1] uncultured bacterium. marine biofouling in heat exchangers | unpublished

[JX984100.1] uncultured bacterium HAMb1_074. biofilm on blue mussel *Mytilus edulis* [HJ799068.1] | unpublished
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Supplementary Figure 1. Physiological responses of in the coral holobiont *Pocillopora verrucosa* in two treatments over time. A. Maximum quantum yield of *Symbiodinium* cells *in hospite*. B. Holobiont-associated gross photosynthetic rates. C. Holobiont-associated dark respiration rates. N = nitrogen. No significant differences were apparent from (gee)glms (p > 0.05).
Supplementary Figure 2. Taxonomy-based functional (metabolic) profiling of bacterial communities associated with the coral holobiont *P. verrucosa* in control versus EN treatments over 14 days. Heatmap created in METAGENASSIST displaying changes in putative functional profiles based on the 16S community composition. Changes are displayed on a relative scale with enrichment in red and depletion in blue. Data were analyzed for metabolism by phenotype with a Pearson distance measure and Ward clustering algorithm. C = control treatment. N = excess nitrogen treatment. 0, 7, 14 = days from start of excess nitrogen manipulation.
**Supplementary Figure 3.** Emergence of ciliates in lesioned tissues of *Pocillopora verrucosa* collected from corals in the EN treatment. A. Overview light micrograph of lesioned coral tissues from EN coral fragments. Small shapes are *Symbiodinium*, while larger aggregates are ciliates (black arrows) after ingestion of algal symbiont cells. C. Detail light micrograph of an individual ciliate after the ingestion of multiple *Symbiodinium* cells.
SUPPLEMENTARY DISCUSSION

Metabolic Profiling and Indicator Taxon Analysis of the Coral-Associated Bacterial Community

The overall coral tissue-associated bacterial community remained structurally and functionally stable despite the EN treatment. This finding contrasts with the probiotic hypothesis and previous reports on distinct bacterial community changes under high nutrient enrichment (Thurber et al. 2009; Reshef et al. 2006). In this context, the reported significant downregulation of metabolic pathways associated with the degradation of organic compounds, specifically saccharides, is particularly interesting. Under EN, lower rates of photosynthates are expected to be translocated to the coral host, as *Symbiodinium* may retain more fixed carbon for their own metabolism (43). As these photosynthates are mostly saccharides (glycerol, glucose) (44), and not all photosynthates are retained within the host (45) reduced rates of translocation to the host may affect its tissue-associated bacterial community.

An increase in bacterial indicator taxa has been reported for white-band diseased (WBD) corals (46) and visibly healthy corals on reefs chronically exposed to poor water quality (18). In the present study, a substantial amount of the indicator OTUs belonged to potentially opportunistic groups, specifically Rhodobacteraceae, Saprospiraceae, Alteromonadaceae, Bacteriovoracaceae, Bdellovibrionaceae, as well as Vibrionaceae, all of which were previously reported to be significantly associated with stressed (Ziegler et al. 2015) or diseased corals (46, 47). Several OTUs (or genetically close matches) were previously associated with diseases of marine organisms, for instance white plague disease (OTU0076, 0539; Sunagawa et al., 2009), as well as *Porites* white patch syndrome (OTU0176, unclassified Alteromonadales; unpublished; GenBank accession number KF180023.1). Notably, the opportunist *Vibrio shilonii* emerged as significant associate under EN conditions. Opportunistic *Vibrio* were previously identified as the causative agents of bleaching in the octocoral *Oculina patagonica* (Rosenberg & Falkovitz 2004) and of disease in *Pocillopora damicornis* (*V. corallilyticus*; (48), and opportunistically occur in stressed scleractinian holobionts (49). Further interesting is the emergence of *Nautella* (OTU0002), which was highly prevalent in the EN bacterioplankton community, but also the
most abundant significant associate in EN-stressed corals after 14 days. One member of this genus, *Nautella* sp. R11, is a known pathogen causing bleaching in the red macroalga *Delisea pulchra* (Fernandes et al. 2011). This emergence may be an indication of the overwhelming of defensive mechanisms in *P. verrucosa*, and reflect pathogen entry from the surrounding seawater. Intriguingly, these opportunistic indicators only occurred after the onset of partial mortality (i.e. tissue lesions) in the presence of an otherwise unaltered overall microbiome, highlighting strong host selective forcing even under holobiont breakdown.
SUPPLEMENTARY REFERENCES


CHAPTER 5

General Discussion
GENERAL DISCUSSION

Overview

This thesis aimed to provide insight to the contribution of microbes to coral holobiont functioning by conducting a set of manipulative experiments featuring high DOC and EN enrichment. The presented work shows that a DOC-induced bleaching phenotype in the coral holobiont is strongly influenced by the activity of coral-associated nitrogen fixing bacteria, and that the functional dependencies of the main coral holobiont members to nitrogen cycling may be strongly driven by host selective forcing in *P. verrucosa*. Finally, the findings of this thesis suggest the existence of different ecological strategies with respect to their dependence on the nitrogen fixation pathway. Hence, this thesis provides interesting new angles on coral stress responses to environmental change.

Halfway through this thesis, a mass coral bleaching event started that would later be dubbed the ‘Third Global Bleaching Event’. At the time of submission of this thesis, the event is still ongoing. It is caused by prolonged and exceptionally high temperatures in reef waters around the world, and was periodically exacerbated by a particularly strong *el Niño* Southern Oscillation. The event started in mid-2014, and is now predicted to globally last until the end of 2016, or even well into 2017 (NOAA). Therefore, the Third Global bleaching is not only the longest-lasting and most severe event in recorded history, but it is impacting some reefs repeatedly in consecutive years. To make the general situation for coral reefs even worse, the beginning of the ongoing bleaching had occurred within less than five years after the Second Global Bleaching Event (2009/10). The current event encompasses an unprecedented bleaching coverage and mass mortality in numerous reef systems all around the planet, from the Red Sea to the Indo-Pacific, as well as the Tropical Atlantic (XL Catlin Seaview Survey). As most reefs struck by coral mass mortality fail to fully recover, the Third Global Bleaching Event will mark an important turning point in coral reef conservation. If not addressed now, the coral reef crisis will ultimately lead to the first modern global extinction of an entire ecosystem (1–3). Consequently, immediate action is needed to implement meaningful conservation practices to mitigate the effects of climate change on corals and their reefs. Any conservation actions however require a detailed understanding of the causes as well as underlying processes of coral reef decline. In this context, there is compelling evidence that local stressors, such as elevated nutrient availability may exacerbate the effects of global stressors on coral reefs by promoting the susceptibility of corals to bleaching (4–7), disease, and, ultimately, mortality (8). This thesis hence offers an important advance in our understanding of the processes involved in the stress response of scleractinian coral holobionts and may provide new opportunities for improved reef
management. Preliminary answers to each of the 4 questions formulated in Chapter 1 are provided below.

1) How does high DOC availability affect coral-associated nitrogen fixation? What are the ecological implications?

This thesis provides a first combined account of nitrogen fixation measurements, diazotroph abundances (Chapter 2, 3), and \textit{nifH} gene expression rates in a comparative taxonomic framework covering four common species in two Indo-Pacific coral families (Chapter 3). Thereby, it demonstrated that diazotrophs and active transcription of the \textit{nifH} gene occur in holobionts not exhibiting detectable nitrogen fixation activity, albeit at low abundances and levels compared to holobionts exhibiting measurable to high rates of nitrogen fixation. Therefore, the association with diazotrophs appears to be inherent with scleractinian holobionts, even though it might not be of equal ecological significance in different species of coral. This thesis concluded that in the investigated coral species, diazotrophs are members of the rare microbiome even in corals associated with high nitrogen fixation activity (Chapter 2, 3), but exhibited marked patterns of abundance and activity conserved at the family level (Chapter 3). Specifically, the Pocilloporidae harbor a rather sizable core microbiome dominated by a single non-diazotroph bacterial family (Endozoicomonaceae) (9–11) (Chapter 4). Diazotroph communities associated with Pocilloporidae are therefore small, but apparently highly active, as reflected in high \textit{nifH} gene expression and nitrogen fixation rates (Chapter 2, 3). In the Fungiidae however, diazotroph abundance and active description of the \textit{nifH} gene was found to be two orders of magnitudes lower than in the Pocilloporidae, and no nitrogen fixation was detectable (Chapter 3). This suggests a low ecological significance of nitrogen fixation in the Fungiidae. Consequently, two distinct nitrogen acquisition strategies may have developed in scleractinian corals: dependence on holobiont-associated nitrogen fixation activity, and the exploitation of other nitrogen sources. As heterotrophy is associated with all scleractinian corals, but heterotrophic capacity (12–14) as well as coral-associated nitrogen fixation vary among species (15, 16) (Chapter 3), the association with one or the other may not be exclusive.

While providing a first comparative taxonomic account of the physiological and molecular basis of nitrogen fixation in scleractinian corals, this thesis covers only four species in two widely distributed coral families (both Clade Robusta; Chapter 3). There is however evidence for the potentially low ecological significance of microbial nitrogen fixing activity in members of at least one more robust coral family, the Merulinidae [Shashar et al. (1994) reported an absence of detectable nitrogen fixation activity in \textit{Hydnophora contignatio}; however,
high nitrogen fixation activity is known from *Goniastrea spp.* (16, 17), and diverse diazotroph communities were reported for *Montastrea cavernosa* (18), both merulinids. The reliance on diazotrophy may thus not be determined by the taxonomy of the host alone, but rather by their heterotrophic capacity for nitrogen acquisition (Chapter 3). These findings warrant further investigation to determine the main sources of nitrogen acquisition in corals not heavily relying on microbial nitrogen fixation, and to extend the investigation of the molecular basis of coral-associated nitrogen fixation in an extended taxonomic framework (Chapter 3).

2) **What is the potential role of nitrogen fixing bacteria in coral bleaching? What are the potential implications of changes in nitrogen fixation activity on the ecosystem level?**

The set of response parameters investigated in this thesis, taking the holobiont, *Symbiodinium*, and the diazotroph community into account, suggests that the stimulation of coral-associated nitrogen fixation under high DOC (and, potentially, elevated temperatures) may rather destabilize than maintain holobiont functioning, and can indeed induce a bleaching phenotype (Chapter 2). Specifically, the uptake of microbially fixed nitrogen by *Symbiodinium* resulted in the nutrient-imbalanced growth of algal symbionts (as reflected in the significant depletion of δ¹⁵N and an increased N:P ratio, respectively), followed by marked decreases in photophysiological properties (Chapter 2). Stoichiometric changes in the seawater nutrient environment, specifically increases in the N:P ratio promote the substitution of phospho- with sulfolipids in the photosynthetic membranes of *Symbiodinium*, thereby increasing the algal symbiont’s susceptibility to heat and light stress. Subsequently, the nutrient-imbalanced coral holobiont displays an overall increased bleaching susceptibility (3). While Wiedenmann et al. (2012) reported lowered bleaching thresholds under heat and light stress, however, the diazotroph-mediated bleaching phenotype observed in Chapter 2 occurred at non-stressful temperatures and light levels.

The suborganismal responses described in Chapter 2 illustrate the breakdown of the coral holobiont under stimulated nitrogen fixation activity. These findings however may have critical implications on the ecosystem level, as the skeletons of recently killed corals are commonly rapidly colonized by a succession of turf and macroalgae (19). Epilithic microbes (20, 21) and turf algae biofilms (22) associated with dead coral skeletons exhibit higher nitrogen fixation rates compared to live holobionts, and exude large amounts of (labile) DOC (23–26) (Chapter 3). Algae-derived labile DOC may therefore further stimulate nitrogen fixation in nearby corals, again inducing bleaching, disease, and, eventually, death. Indeed, the detrimental effects of algal exudates on corals have been demonstrated elsewhere (27–29). On reef scale, this could potentially result in considerable positive feedback loops of ecosystem degradation (19,
In this context however, more research will be required to tease apart the effects of algae-derived DOC and cytotoxic allelochemicals which are both components of soft coral mucus (31) and algae exudates (32).

3) How is holobiont breakdown manifested under high DOC and EN provision? Is there evidence for a functional dependency of the individual members of the holobiont on nitrogen cycling?

This thesis provides a preliminary account of the responses of the holobiont, coral, *Symbiodinium*, and coral-associated bacteria to changes in water quality (Chapter 2, 4). Specifically, both treatments aimed to overwhelm the host-regulated nitrogen limitation via EN availability. This was achieved by the stimulation of coral-associated nitrogen fixation (internal EN availability, indirectly via high DOC provision; Chapter 2), or via eutrophication (direct external EN availability; Chapter 4). While the high DOC treatment increased the internal availability of microbially fixed ammonium (Chapter 2) [ammonium is the product of microbial nitrogen fixation (33)], labile DOC can induce coral bleaching, disease, and mortality, which has previously been attributed to increased bacterial growth and respiration, and the subsequent formation of oxygen-depleted layers on the coral surface (27, 34–36), as well as virulence gene expression (37). While at this point direct and potentially detrimental effects of high labile DOC cannot entirely be ruled out, no significant treatment effects on the overall bacterial community composition and bacterial abundance were apparent in general, and no propagation of potentially opportunistic groups such as Vibrionaceae was observed in the tissues *P. verrucosa* (Chapter 2). Despite this absence of shifts in the overall bacterial community composition however, this thesis was able to demonstrate that DOC can indeed fuel the proliferation and activity of coral-associated diazotrophs [which appear to be members of the rare biosphere, as evident from 16S rRNA data and the overwhelming dominance of *Endozoicomonas*], allowing insight into the significance of stimulated nitrogen fixation activity for holobiont functioning (Chapter 2, 3). Consequently, stimulated nitrogen fixation may also have been fueled by high DOC availability and oxygen-depleted conditions and contributed to the detrimental and (sub)lethal effects reported by previous studies (i.e., Kuntz et al. 2005; Kline et al. 2006; Smith et al. 2006; Haas et al. 2009; Thurber et al. 2009). However, the potential harmful role of stimulated nitrogen fixation in holobiont functioning and breakdown will have to be addressed in a comparative coral taxonomic framework.

*Symbiodinium* are strictly nitrogen limited by the host (38, 39) and take up microbially-fixed nitrogen (Lesser et al., 2007; Olson et al., 2009; Cardini et al., 2015) (Chapter 2). Therefore, the algal symbionts were expected to exhibit a strong functional dependency to changes in coral
nitrogen cycling due to EN availability. This functional dependency was remarkably
demonstrated in dramatic physiological and numerical responses of the algal symbionts,
resulting in the rapid breakdown of the coral-algae symbiosis (i.e., within 14 to 28 days). The
phenotypes of symbiotic breakdown however were distinct in holobionts subjected to different
treatments (Chapter 2, 4). Increased coral-associated nitrogen fixation in the high DOC
treatment resulted in a bleaching phenotype in the absence of heat and light stress (Chapter 2),
which was not observed under EN availability (Chapter 4). The disparity between the bleaching
and eutrophic phenotype of symbiotic breakdown described in this thesis could likely be
explained by confounding effects associated with a) the detrimental effects of high DOC
availability itself (e.g., hypoxia, virulence gene expression; Kline et al. 2006; Smith et al. 2006;
Thurber et al. 2009), and b) a co-enrichment of various nitrogen species in the EN treatment due
to the photodissociation of urea into carbon dioxide and ammonium, and degradation processes
downstream (40) (Chapter 4). Indeed, the differential enrichment of nitrogen species appears to
affect the actual rates of Symbiodinium (in)organic carbon acquisition, fixation, and translocation
to the host (Ezzat et al. 2015).

Both treatments were associated with rapid (i.e., within 7 days of treatment in both
manipulations) differential changes in Symbiodinium cell density and effects on (photo-)
physiology. The high DOC bleaching phenotype of symbiotic breakdown exhibited a significant
loss of algal symbionts and drops in Gp and the maximum quantum yield, as well as increased R
(Chapter 2). This uncommon combination of responses [thermally bleached corals exhibit
decreased R, as less photosynthate is translocated from stressed Symbiodinium to the host (41)]
can likely be attributed to a utilization of DOC by the coral host in combination with increased
microbial growth and respiration (27, 34) as well as induced photodamage to the algal
symbionts (4). In contrast, corals in the eutrophication (EN) treatment harbored increased
Symbiodinium densities and cell chlorophyll a content. While no statistically significant effects on
photosynthetic properties (unless normalized to individual algal symbiont cells) and R were
apparent, holobiont dark calcification was dramatically decreased at higher Symbiodinium
densities, and the resident Symbiodinium population exhibited marked compositional
fluctuations in hospite under EN availability. Reduced calcification despite an absence of
detrimental effects on coral photophysiology may be explained by the increased retention of
photosynthates within the algal symbiont cells, decreasing the overall amount of organic carbon
translocated to the coral host (42). Nitrogen availability however not only regulates the density
of the Symbiodinium population, but apparently can also affect its taxonomic composition, as
reflected in the almost complete loss of one of the clades dominating P. verrucosa (Chapter 4). As
the remaining clade A1 is highly prevalent along almost the entire Red Sea basin (Sawall et al.
2014), these findings suggest a superior nitrogen uptake capacity of A1 compared to D6. Indeed,
Symbiodinium clade D have previously been reported to exhibited lower nitrogen uptake rates compared to other clades at ambient temperatures (43, 44).

While the overwhelming of host-regulated nitrogen limitation via internal ammonium provision [via the stimulation of holobiont-associated nitrogen fixation; Chapter 2] as well as external EN provision resulted in the derailment of the coral-algae symbiosis (Chapter 4), it did not affect the overall bacterial community composition in the pocilloporid coral P. verrucosa over 28 and 14 days, respectively. Under EN conditions, an emergence of very rare indicator taxa (i.e., taxa significantly associated with a treatment or condition) was apparent 24 hours after the onset of massive tissue lesions at 13 days, i.e. once partial host mortality had set in (Chapter 4). Even then, however, the overall bacterial community structure did not exhibit significant treatment responses, likely due to the overwhelming dominance of its core microbiome members. As both DOC and EN (or nutrients in general) may cause an increased susceptibility of corals to bleaching, disease, and mortality (4–6, 45, 46) and are associated with shifts in the bacterial community composition in various coral species (10, 37, 47), the results presented here appear to be contradictory at first. While this suggests the bacterial community associated with the P. verrucosa holobiont does not exhibit a functional dependency on nitrogen cycling, the questions arise: how, and why? For a detailed discussion, see Chapter 4, and below.

4) Is there evidence for host regulation / forcing on the Symbiodinium, bacterial, or diazotroph communities in the P. verrucosa holobiont under high DOC and EN availability? What are the potential implications of diazotroph dependency and a highly selected holobiont composition for stress susceptibility and acclimatization in P. verrucosa in a changing world?

The mutualistic coral-algae symbiosis is characterized by complex interactions of the two partners (38, 48, 49). Particularly the nitrogen limitation of Symbiodinium by the coral host is considered one of the main regulatory mechanisms underlying the coral-algae symbiosis (50, 51). This thesis confirmed the uptake of excess microbially fixed nitrogen by Symbiodinium (as evident in the simultaneous increase of the algal symbiont N:P ratio and depletion of δ15N) following the overwhelming of host-imposed nitrogen limitation via stimulated diazotrophy, and reported cascading effects on holobiont physiology and functioning downstream (Chapter 2). In contrast, while no uptake of microbially fixed nitrogen by the coral animal was apparent (i.e., no decrease in the δ15N signature), the dramatic increase of the coral tissue N:P ratio suggests the coral host attempted to counteract the EN availability by restoring nitrogen limitation and thereby the coral-algae symbiosis (Chapter 2). Based on this significant increase in the coral tissue N:P ratio, potential regulative buffering mechanisms could involve the physical separation
of nitrogenous compounds from *Symbiodinium* in specialized cellular structures or organelles located in the coral tissue, as previously suggested for the storage of assimilated ammonium (52). While any putative attempts to restore nitrogen limitation by the holobiont appeared to fail preventing symbiotic breakdown in this thesis (Chapter 2), in the bigger scheme, this postulated mechanism would be in full accordance with previous studies on *Symbiodinium* population control (38). Further investigation will be required to identify the nature of the postulated buffering mechanisms, whether those are inherent to scleractinian corals in general, and whether they are similarly employed during thermal stress and bleaching.

Evidence for strong selective forcing by the coral host was further observed in the maintenance of a stable bacterial community under high DOC and EN conditions, and even after the onset of holobiont breakdown in *P. verrucosa* in the latter (Chapter 2, 4). This was apparent at the overall bacterial community level, and reflected in the abundance of diazotrophs. First, the overall bacterial community associated with the *P. verrucosa* holobiont did not exhibit significant compositional changes under high DOC and EN nitrogen availability. While significant associations occurred exclusively in the rare microbiome (i.e., 40 indicator taxa with a cumulative contribution of 0.15 % to the overall number of sequences) in EN corals at 14 days, these changes did not occur before 24 h after the onset of massive tissue lesions, i.e. partial host mortality (Chapter 4), suggesting strong host selective forcing to maintain a stable bacterial community even after holobiont breakdown ensues. Second, while the diazotroph community associated with *P. verrucosa* (Pocilloporidae) and *P. granulosa* (Fungiidae) responded to high DOC provisions with increases in abundance (Chapter 2, 3) and increased rates of nitrogen fixation activity or *nifH* gene expression, respectively (Chapter 2, 4), they differ with respect to the temporal scale. The *P. granulosa* holobiont, not fixing nitrogen at detectable (and therefore probably not ecologically significant) rates (Chapter 3), but displaying a rather flexible microbiome (53), exhibited ‘immediate’ diazotroph proliferation in two high DOC treatments over 24 h (exposition to 4 and 40 ppm DOC; Chapter 3). In contrast, while *P. verrucosa* exhibited significantly stimulated nitrogen fixation within 7 days of a high DOC treatment (15 ppm), diazotroph proliferation did not occur before 28 days (DOC; Chapter 2). This temporal lag further substantiates the view that strong host selective forcing must be at bay to maintain a stable bacterial community in the *P. verrucosa* holobiont, which was only overwhelmed during holobiont breakdown (Chapter 4).

Tropical scleractinian corals are highly sensitive to a range of anthropogenic stressors, specifically the effects of global climate change and local stressors (2, 54). In a comparative taxonomic framework, however, marked differences in stress susceptibility are apparent (54). In the light of the findings of this thesis, two mechanisms potentially driving stress susceptibility in corals are postulated. These putative mechanisms include a) diazotroph dependency (Chapter 2,
4), and b) holobiont selection (Chapter 2, 4). Coral-associated nitrogen fixation is considered beneficial for coral holobiont functioning due to the provision of new fixed nitrogen (55, 56, 15), suggesting a potentially high dependency on this functional trait in oligotrophic coastal waters (Chapter 1). Similar to eutrophic conditions (Chapter 4), microbially fixed EN availability however may rapidly derail the coral-algae symbiosis (19) (Chapter 2), even though the phenotypes of symbiotic breakdown vary (Chapter 2, 4). In this context, this thesis compared physiological and molecular nitrogen fixation properties in two families of tropical scleractinian corals characterized by marked differences in stress susceptibility. The weedy Pocilloporidae are widely distributed throughout the Indo-Pacific (57), but highly susceptible to the effects of ocean warming (54, 57, 58). In contrast, the Fungiidae exhibit a high resistance to various stressors (59–63), including ocean warming (54, 64). Notably, the differential stress susceptibility in Pocilloporidae and Fungiidae align with family-level patterns in their nitrogen fixation properties (Chapter 3) [and potentially the flexibility of their respective bacterial communities (Chapter 4; 53)]. While more empirical data are required to fully understand the significance of the functional trait nitrogen fixation in corals, the preliminary results of this thesis suggest that this process could render the holobiont more susceptible when stimulated by certain environmental stressors, and thereby be a driver of coral and reef resilience (Chapter 2, 3).

The Coral Probiotic Hypothesis postulated the acclimative restructuring of coral-associated microbial communities under changing environmental conditions within days to weeks (65). While this acclimative restructuring has been reported by only few studies (10, 53), rather the opposite was observed in this thesis. Indeed, a marked invariance of the bacterial communities associated with the *P. verrucosa* holobiont became apparent in a high DOC and an EN regime (Chapter 2, 4). This suggests the existence of strong host regulative forces to maintain highly selected *P. verrucosa* holobionts. These selected holobionts appear to be generalist ‘prototypes’ that may perform best in a small range of environmental conditions only, but are sufficiently successful to prevail in a wide geographic range (Chapter 4), for instance the Red Sea basin (66). In the long term, however, the results presented in this thesis suggest that *P. verrucosa* has a rather limited acclimative capacity which may impede its resilience to the effects of global environmental change (Chapter 2, 4).
FUTURE PERSPECTIVES

This thesis provided new insights into the functional importance of nitrogen cycling in scleractinian coral holobiont functioning in the presence of a widely underinvestigated stressor, DOM (in the form of DOC and EN/DON). While the findings presented in this thesis will provide a foundation for future research questions to build and expand on, nitrogen cycling in corals is highly complex, and many more aspects remain elusive. Therefore, more research is warranted to further validate insights from these studies. First and foremost however, it will be of critical importance to translate the knowledge derived from these studies into meaningful management actions. Importantly, this thesis has shown that due to the strong effects on nitrogen fixation and/or overall coral holobiont functioning, nutrient input (DOM and EN/DON) onto coral reefs deserves our immediate attention. Albeit resulting in markedly different phenotypes of symbiotic breakdown, both DOC and EN/DON have a strong capacity to disrupt nitrogen limitation and thereby induce holobiont breakdown and mortality (Chapters 3, 4). While this thesis did not compare the immediate risk of different labile DOC sources (e.g., autochthonous algae and soft coral exudates vs. allochthonous input via sewage release) for overall coral health and performance, significant effects on physiological and molecular properties of the coral-associated nitrogen fixation pathway were apparent regardless the origin of the DOC (Chapter 2, 3). This suggests that any (non-scleractinian) source of labile DOC can potentially affect coral-associated nitrogen fixation, and incapacitate holobiont breakdown, confirming previous results (27, 34) from a nitrogen cycling view. Thereby, one ultimate goal should be to reduce the input of DOC and EN into sensitive coastal waters. This can be achieved by improved waste water treatment procedures (to eliminate the input of allochthonous DOC) in combination with managing benthic coral reef communities, i.e., control the cover of coral reef macroalgae and soft corals (to eliminate the input of non-scleractinian, autochthonous DOM). While more research is needed at this point, the results of this thesis suggest that management of (in)organic nutrient levels on coral reefs should be prioritized to aid in the mitigation the effects of global climate change.

As scleractinian corals appear to be associated with possibly complete nitrogen cycles (67–69), it will be critical to implement holistic and direct efforts to quantify the individual pathways as well as the associated microbial players in combined approaches of physiological and culture-independent applications (e.g., (meta-)genomic and expression-based studies). In this context, it will further be interesting to delve deeper into the potential link between nitrogen fixation activity and coral holobiont resilience by investigating the role of phosphorus availability in nitrogen cycling and, ultimately, holobiont functioning, and comparing diazotroph dependence (Chapter 3) as well as holobiont selection (Chapter 4) in a broad taxonomic framework subjected to manipulative future ocean scenarios.
Finally, cellular level approaches will be required to fully understand the mechanisms underlying the complex inter-kingdom relationships within the coral holobiont. In this context, high resolution single cell imaging based on secondary mass ion spectroscopy (SIMS) approaches allow for the examination and characterization of ions and complex molecules directly from biological materials on the nanometer scale (70, 71). Stable isotope labeling techniques coupled with NanoSIMS technology are emerging applications in functional coral studies to determine the fate of (nitrogenous and other) metabolic products in different functional compartments of the coral holobiont (30, 52, 72, 73). Similarly, while not (yet) established for functional coral physiology studies, a combined application with Time-of-Flight techniques (ToF-SIMS) to determine the molecular composition from the surface of biological materials (74) may provide critical insight into holobiont nitrogen cycling. These applications will be particularly interesting to elucidate the putative buffering mechanisms under stimulated nitrogen fixation activity postulated in this thesis (Chapter 2).

CONCLUSIONS

This thesis showed that two widely underinvestigated anthropogenic stressors, specifically DOC and EN (via DON provision), can rapidly disrupt coral holobiont functioning. These detrimental effects were in general attributed to a functional dependency of the holobiont to internal nitrogen cycling, and specifically to changes in the nitrogen fixation pathway. Thereby, this thesis has made significant contributions to our understanding of coral holobiont functioning, complex underlying inter-kingdom interactions, and how these may shape the coral reefs of the future. Holobiont-associated microbial nutrient cycling pathways, specifically nitrogen fixation, rapidly respond to anthropogenically-driven environmental change, thereby derailing the coral-algae symbiosis and ultimately leading to holobiont mortality. The findings presented in this thesis further suggest that on impacted and degraded reefs, (microbial) feedback loops acting on the ecosystem scale, and, exacerbated by the differential activity of microbial processes, may further promote coral reef decline.
REFERENCES


Appendix:

Review

Nitrogen cycling in corals: the key to understanding holobiont functioning?


The video abstract can be found at: https://www.youtube.com/watch?v=DWIItFGRQJ4
Corals are animals that form close mutualistic associations with endosymbiotic photosynthetic algae of the genus *Symbiodinium*. Together they provide the three-dimensional calcium carbonate framework of coral reef ecosystems. Only recently, the importance of the microbiome (i.e. bacteria, archaea, fungi, and viruses) to holobiont functioning has been recognized. Given that growth and density of *Symbiodinium* within the coral host is highly dependent on nitrogen availability, nitrogen cycling microbes may be of fundamental importance to the stability of the coral–algae symbiosis and holobiont functioning, in particular under nutrient-enriched and -depleted scenarios. Here, we summarize what is known about nitrogen cycling in corals and conclude that disturbance of microbial nitrogen cycling may be tightly linked to coral bleaching and disease.
INTRODUCTION

Corals in an oligotrophic environment

Tropical reef-building corals commonly flourish in nutrient-poor environments. The contradiction of high coral productivity on the one hand and limited nutrient availability on the other hand has been coined the ‘Darwin Paradox’ in reference to its first observer (1, 2). The highly efficient uptake and recycling of nutrients in coral reef organisms can help to explain this paradox (3, 4). Particularly for corals, the close association between the coral animal host and its endosymbiotic dinoflagellate algae of the genus *Symbiodinium* enables an effective use and retention of nutrients and photosynthates, i.e. photosynthetically fixed carbon (5). Together with their algal symbionts, corals are associated with a variety of other microorganisms, including protozoans, fungi, archaea, and bacteria (6, 7), an assemblage termed the coral holobiont (see Glossary). The resulting meta-organism represents a complex interactive system with the potential to extend the physiological capabilities of the coral host (8). Hence, knowledge of underlying mechanisms and interactions within the holobiont framework is essential to comprehend the response of corals to environmental change, such as ocean acidification, ocean warming, and eutrophication.

Distinct microbial assemblages are associated with the surface mucus layer, coral tissue, gastro-vascular cavity, and within the coral skeleton (8). This variety of habitats results in a highly diverse coral microbiome where associated microbes can potentially perform a multitude of services to the functioning of the coral holobiont including carbon fixation, nitrogen metabolism, sulphur cycling, and anti-microbial defence among many others (6, 9–11). Due to the high importance of different microbial groups for the functioning of the coral holobiont, a variety of mechanisms have evolved to enable an effective intergenerational transfer of specific microbes to ensure offspring fitness (12, 13). This vertical transfer presumably facilitated the evolution of host-specific and persistent microbial communities in many corals (14–18). In this context, Reshef et al. (19) proposed the coral probiotic hypothesis, stating that despite the presence of consistent microbial communities, alterations in the microbiome may help corals to rapidly adjust to environmental conditions (Box 1).

In particular, nitrogen cycling microbes appear to be ubiquitous and consistent members of the coral microbiome (8, 9, 16, 17, 20, 21). Given that symbiont production in corals is highly dependent on nitrogen availability (22, 23), nitrogen cycling in the coral holobiont among other factors may be critical for acquisition and retention of nitrogen to sustain primary productivity (i.e. photosynthesis).
In this review, we thus summarize the current knowledge of microbial nitrogen cycling within the coral holobiont and its importance for the coral–algae symbiosis. Furthermore, we discuss potential effects of environmental change on these pathways with a focus on their putative role in the occurrence and deleterious effects of coral bleaching and disease.

**Box 1. The coral probiotic hypothesis**

Corals harbour a variety of symbiotic archaea and bacteria. Changing environmental conditions can alter the composition and abundance of coral-associated microorganisms rapidly. Reshef & colleagues (19) proposed in their “coral probiotic hypothesis” that a dynamic relationship between symbiotic microorganisms and the coral host selects for the most advantageous composition of the coral holobiont under varying environmental conditions. Therefore, shifts in the microbiome may facilitate corals to adjusting to changing environmental conditions considerably faster than by mutation and selection of the host alone. This implies that the combined holobiont rather than its individual members represents the unit of natural selection, and has led to the development of the “hologenome theory of evolution” (8).

Some studies suggest that environmental changes acting on the coral holobiont may select for partners, which are beneficial for coral holobiont functioning (94, 95). Particularly the development of anti-microbial resistance in corals, despite the lack of an adaptive immune system, highlights the adaptive potential of microbial interactions within the holobiont (96).

It has been shown that environmental conditions can affect nitrogen cycling in corals [46], and that nitrogen fixation in soft corals can supplement reduced nutrient availability in the Red Sea (97). It is likely that these alterations in nitrogen cycling capacity are the results of shifts within the coral microbiome. Hence, in accordance with the coral probiotic hypothesis, these changes may serve as a way for corals to adapt to changing nutrient availability.

**Nitrogen uptake and symbiont control in the coral holobiont**

The coral holobiont is highly efficient in the assimilation of nitrogen. Heterotrophic feeding by the coral can meet a large part of its nitrogen requirements if sufficient food is available (24). In addition, corals acquire nutrients from their symbiotic algae from the genus *Symbiodinium*. This symbiotic relationship forms the foundation of coral holobionts and shallow-water coral reefs. In this association, the phototrophic dinoflagellates provide photosynthates to the coral host. However, the translocated photosynthates have been referred to as "junk food" as they show a high C:N ratio and therefore require additional nitrogen supplementation to sustain coral growth (25). The symbiotic algae benefit from inorganic nutrients which are released as
metabolic waste products by the host (5, 26). Their capacity for efficient uptake and utilization of dissolved inorganic nitrogen (DIN) facilitates the acquisition of nitrogen from the surrounding seawater. Although both the coral host and associated *Symbiodinium* have the enzymatic machinery to incorporate ammonium, the algae account for most of the uptake of dissolved inorganic nitrogen from the environment, mainly in the form of ammonium (NH$_4^+$) and nitrate (NO$_3^-$) [27]. This nitrogen, together with host-derived nitrogen compounds, is either stored by the algae or used in their metabolism and may be partially translocated to the coral host in form of organic nitrogen compounds, e.g. amino acids (27–30). Together with an efficient recycling of coral metabolic waste products within the holobiont, this symbiosis thus enables efficient utilization of nitrogen compounds from surrounding seawater.

The availability of nitrogen sources in coral reefs however underlies strong seasonal and diel variations and can be impacted by anthropogenic activities (31). Consequently, internal regulation mechanisms need to be in place in order to control for these fluctuations.

At least three mechanisms have been identified by which corals exert control over their algal symbionts: (i) compounds of the coral host tissue, so-called host release factors (HRFs), trigger the release of photosynthetically fixed carbon in freshly isolated *Symbiodinium* (32–34). These HRFs are likely specific amino acids and crucial to ensure the nutrition of the coral host and to increase photosynthetic rates (via unknown mechanisms). (ii) Control of algal numbers by degradation/digestion (35). Via this process the host may benefit from the organic nutrients contained in the symbionts. (iii) Control of *Symbiodinium* density by limiting nutrient availability (22). Nitrogen limitation in particular may be essential to regulate cell division rates of the faster proliferating *Symbiodinium* to match those of the host (36, 37). Furthermore, the translocation of photosynthates to the host can help the symbionts to maintain a favorable carbon to nitrogen ratio, since carbon fixation by the algae proceeds in particular in high light environments despite nutrient limitation (38). Wiedenmann et al. (39) showed that a shift away from nitrogen limitation by excess nitrogen provision can ultimately results in phosphate starvation, which can increase the susceptibility of corals to a heat and light stress-mediated loss of their algal symbionts (coral bleaching). Hence, low internal nutrient availability, specifically of nitrogen, seems crucial to maintain high primary production, while simultaneously controlling algae growth.

In this context, adjustment of internal nitrogen availability may help to control algal growth. The ability of the coral host to assimilate ammonium has been suggested to allow for a more efficient uptake of ammonium from seawater by maintaining the diffusion gradient into the tissue, whilst simultaneously enabling an internal limitation of nitrogen availability to *Symbiodinium* (27, 36, 40). At the same time, regulation of symbiosome pH surrounding *Symbiodinium* may help to control the diffusion of ammonium ions into the symbiosome (41).
Furthermore, nitrogen cycling by coral-associated microbes may be an additional mechanism contributing to the stabilization, or conversely destabilization, of the coral–algae symbiosis.

**Nitrogen fixation**

Coral reefs are net sources of fixed nitrogen (42). Nitrogen fixation, i.e. the conversion of elemental dinitrogen (N₂) into ammonium, is associated with many substrates (e.g. sand, coral rock, and rubble) and benthic organisms (e.g. corals, macroalgae, and sponges) (21, 43). This input of new fixed nitrogen into the reef ecosystem helps to sustain net productivity under oligotrophic conditions and to compensate for net nitrogen export from the system, for instance, by currents (44). Nitrogen fixation in hermatypic corals has been reported for several different species (45–48), suggesting a high relevance of this process for the coral holobiont. However, reported nitrogen fixation rates in corals are about a magnitude lower than those found in reef sediments and bare rock (43). Hence, corals appear to be minor contributors to the overall nitrogen budget of coral reefs.

For a long time, cyanobacteria were believed to be the main drivers of nitrogen fixation in corals (49–51), but recent studies revealed that corals harbor ubiquitous diverse communities of diazotrophs, i.e. nitrogen-fixing bacteria and archaea, consisting of mostly heterotrophic bacteria (16, 17, 20, 52). These communities are coral species-specific and highly persistent over space and time (16, 17). Moreover, Lema et al. (18) found a vertical transfer of diazotrophs from parental colonies of the coral *Acropora millepora* to their larvae, mostly Alphaproteobacteria of the group Rhizobiales. This vertical transfer of diazotrophs further suggests a beneficial role of this group for holobiont functioning. Although the magnitude of transfer of fixed nitrogen from diazotrophs into other compartments of the coral holobiont (e.g. *Symbiodinium*) has not been quantified yet, recent studies show that bacterial symbionts contribute to the nitrogen supply of the holobiont (48, 53). However, a recent stable isotope (¹⁵N₂ gas) tracing application in Red Sea corals revealed no direct incorporation of fixed nitrogen in the coral tissue within the first 24 hours of measurement, suggesting coral–diazotroph interactions may be more complex than previously thought (47).

Nitrogen fixation is a highly energy-consuming process, which requires 16 mol of ATP for the reduction of 1 mol of dinitrogen (54). Therefore, nitrogen fixation is energetically more costly than other mechanisms of ammonium assimilation. Hence, other sources of fixed nitrogen may be favored over nitrogen fixation, if available. The depressing effect of combined nitrogen availability on nitrogen fixation by terrestrial, planktonic, and benthic diazotrophs has been reported (55). It is thus likely that similar regulatory mechanisms to reduce nitrogen fixation rates at times of excess nitrogen availability exist in coral-associated diazotrophs (Figure 1). Nitrogen fixation activity in corals is highly dynamic and can be rapidly affected by changes in
environmental conditions (46, 48). Consequently, nitrogen fixation may serve as a mechanism to counteract shortages of environmental nitrogen availability, and maintain a constant nitrogen supply for symbiont-based primary production in corals. This view is further supported by the findings of Olson et al. (52) and Lesser et al. (48), who reported a positive correlation of diazotroph abundance with density and DNA content of *Symbiodinium* cells.

Despite the overall comparatively small contribution to the nitrogen budget of the coral holobiont, nitrogen fixation may be still essential to the stability of the coral–algae symbiosis. Consequently, it may constitute an important functional link between carbon and nitrogen fixation within the holobiont and thus contribute to the success of corals in highly oligotrophic reef environments.

**Nitrification**

In contrast to nitrogen fixation, other pathways of the nitrogen cycle in corals have received little attention to date. Nonetheless, particularly nitrification and denitrification may potentially be of importance for holobiont functioning. High rates of nitrification, i.e. the oxidation of ammonium into nitrite (NO$_2^-$) and nitrate (NO$_3^-$), have been measured in coral reef environments (42, 56). Nitrification associated with corals occurs in their skeletons (57), living tissues (58), mucus (59), and the interstitial waters of coral branches (60), likely rendering nitrification a ubiquitous coral-associated process. Consequently, recent studies revealed that nitrifying bacteria and archaea are highly abundant in the microbiomes of many coral species (10, 59, 61, 62). Among these at least mucus-associated ammonia-oxidizing archaeal communities appear to depend rather on location and environmental conditions than the coral host (59, 63). The metabolic activity of these communities however remains yet to be determined, and the role of nitrification in the holobiont is largely unknown. *Symbiodinium* prefer uptake of ammonium over other forms of DIN (64, 65), and ammonium may inhibit the uptake of nitrate (66). Nitrification in corals may thus ultimately reduce the amount of nitrogen available for *Symbiodinium* growth. Wafar et al. (58) reported that bacterial nitrification almost exclusively utilized ammonium derived from coral metabolism and that this process occurred at rates effectively competing with the autotrophic uptake of ammonium. Consequently, nitrogen cycling may serve as a way to retain nitrogen within the coral holobiont, with nitrification preventing loss of ammonium from the holobiont.

**Denitrification**

Depending on environmental conditions, reef substrates can show high denitrification rates, and denitrification has been detected in marine invertebrates (67, 68). Although denitrifying
microorganisms are present in coral microbiomes (10, 62), no studies have investigated the denitrifying potential of corals so far. Siboni et al. (59) suggested a coupling of nitrification and denitrification (i.e. the reduction of nitrate) ultimately resulting in the production of dinitrogen to remove nitrogen from the coral holobiont. Nitrogen cycling processes are highly dependent on oxygen availability (48, 69). Since oxygen concentrations in coral tissue show strong diel fluctuations, the linkage of aerobic (e.g. nitrification) and anaerobic (e.g. nitrogen fixation and denitrification) processes within the coral holobiont may be possible (70). Additional evolutionary adaptations by the coral host and associated microbes may provide a spatial or temporal separation of anaerobic processes from oxygen evolution (71).

Corals can live successfully at a wide range of nutrient concentrations, ranging from highly oligotrophic to eutrophic conditions (31). In addition to regulated nutrient transport within the holobiont, denitrification in combination with nitrification may thus help corals to survive elevated nutrient concentrations and to maintain internal nitrogen-limitation of Symbiodinium at the same time (Figure 1). Future research should therefore aim to identify the acclimation capacity of corals to anthropogenic nutrient enrichment by nitrification and denitrification processes.

Figure 1. Hypothesized nitrogen cycling in the coral holobiont. Representation of major nitrogen cycling pathways in the coral holobiont under (A) oligotrophic, (B) eutrophic, and (C) an elevated temperature scenario. Bold arrows indicate potential relative amplification of pathways compared to an oligotrophic scenario. Dashed arrows indicate potential relative diminution of pathways compared to an oligotrophic scenario. Microbial nitrogen cycling may help to regulate algal growth under oligotrophic and eutrophic conditions, but may lead to elevated nitrogen availability under heat stressed conditions. A representation of the localization of individual process within the holobiont was omitted, as sufficient knowledge is lacking to date. Abbreviation: org., organic.
Other pathways in the nitrogen cycle

Additional nitrogen cycling pathways exist in the holobiont. Wegley et al. (9) suggested that endolithic fungi may exert an important functional role by reducing nitrate into ammonium. These processes enable a highly efficient internal nutrient cycling and thereby may help to prevent the loss of nitrogen from the holobiont. Current knowledge of coral-associated eukaryotic diversity is mostly limited to insights from metagenomic surveys (9, 72). New studies, e.g. using via 18S amplicon based approaches, may provide further clues to eukaryotic organisms associated with the coral holobiont and the services they may provide.

Another pathway of the nitrogen cycle is anaerobic ammonium oxidation (ANAMMOX), transforming fixed nitrogen into elemental dinitrogen. Particularly in the marine nitrogen cycle, the ANAMMOX pathway is of high importance (73). Its presence has been previously confirmed in sponges (68) and has been suggested in corals, but direct evidence still needs to be provided. The presence of ANAMMOX may constitute another critical mechanism besides denitrification in removing excess nitrogen from the coral holobiont, e.g. during eutrophication events.

Environmental constraints and anthropogenic impact

Although nitrogen cycling in corals appears to be of high relevance for holobiont functioning, little is known about the effects of anthropogenic environmental change on this cycle on the holobiont level. Nitrogen cycling may mitigate or exacerbate the impact on corals by channeling nitrogen through different pathways depending on the type of alteration (e.g. global warming or ocean acidification).

Coral reefs have seen an unprecedented global decline over the past decades (74). Particularly coral bleaching and disease are among the main drivers of the loss of coral reef cover (75). Both, bleaching and coral diseases involve the disruption of the coral–algae symbiosis (76). Remarkably, shifts in coral-associated microbial communities, including nitrogen cycling microbes, have been shown to precede the visual symptoms of bleaching and diseases (15, 72, 77–79). Although this correlation does not imply causality and alternative explanations are possible, we investigate a potential mechanism, by which environmental effects on coral-associated nitrogen cycling capacity may be linked to the disruption of the coral-algae symbiosis in the following paragraph.

A potential link between nitrogen cycling and reef degradation

Several studies revealed that nitrogen fixation activity in corals strongly depends on environmental conditions (45, 46, 78). Elevated temperature and dissolved organic carbon (DOC) availability can potentially increase nitrogen fixation activity in corals (Figure 1) (45, 78).
Coincidentally, these same two stress factors are considered strong drivers of bleaching and diseases in corals (40, 80–82).

An imbalanced nutrient availability, i.e. elevated inorganic nitrogen concentrations in combination with phosphate depletion, rather than enrichment of both nitrogen and phosphate, can destabilize the coral–algae symbiosis (39). Among other processes, nitrogen fixation can potentially increase the N:P ratio in corals (31). Hence, environmental impacts that increase nitrogen fixation may ultimately disrupt the nitrogen limitation of *Symbiodinium* in corals. The resulting elevated nitrogen availability would stimulate cell division of *Symbiodinium*, thereby shifting *Symbiodinium* from nitrogen towards phosphate limitation/starvation.

Wiedenmann et al. (39) found that phosphate starvation associated with increased cell division rates resulted in an increase of sulfo- to phospholipid ratios in *Symbiodinium*, which may cause a destabilization of their thylakoid membranes and explain the lower bleaching threshold of phosphate-starved coral species. Furthermore, Wooldridge (40) proposed a mechanism by which enhanced retention of photosynthates by *Symbiodinium* due to excess nitrogen availability may cause carbon dioxide (CO₂) limitation ultimately resulting in coral bleaching. Therefore, environmental stressors such as heat stress and elevated DOC concentrations may potentially lower the threshold at which a disruption of the coral–algae symbiosis occurs by increasing coral-associated nitrogen fixation rates (Figure 2).

Indirect empirical support in favor of this theory is provided by Godinot et al. (83), who reported increased phosphate uptake and net inorganic nitrogen release by the coral *Stylophora pistillata* during heat stressed conditions. This suggests that shifts in the N:P ratio can occur at elevated temperatures. Additionally, increased cell division rates (mitotic index) of *Symbiodinium* during heat stress and bleaching events have been reported, implying a disruption of internal nitrogen limitation of *Symbiodinium* growth (84, 85).
Figure 2. The proposed role of nitrogen fixation in reef degradation. (A) Elevated concentrations of dissolved organic carbon (DOC) and heat stress induce increased microbial activity in corals and stimulate nitrogen fixation activity. (B) The resulting excess supply of fixed nitrogen triggers rapid population growth of the endosymbiotic dinoflagellate *Symbiodinium*. (C) The resulting shift from nitrogen limitation to phosphate starvation in combination with increased *Symbiodinium* population sizes causes a lowered bleaching threshold of corals and eventually coral bleaching. (D) Following colony die-off, the remaining coral skeleton may be overgrown by algae, which release DOC-rich exudates. Hence algae may stimulate further microbial activity including nitrogen fixation in adjacent corals, thereby exacerbating the reef degradation process.

Therefore the proposed pathway may be of importance, not only to better understand and predict coral bleaching events, but also to reveal the underlying mechanisms of coral diseases, which commonly involve the breakdown of the coral–algae symbiosis.

At the same time, breakdown of the coral–algae symbiosis and potential subsequent demise of the coral host may trigger a positive feedback loop of coral degradation (Figure 2), which may act on different scales from colony to ecosystem level (Figure 3). Epilithic algae communities on freshly killed corals show increased nitrogen fixation rates and at the same time release DOC-rich exudates (86, 87). These exudates may stimulate microbial activity
(including nitrogen fixation) in nearby corals. Thus, algae overgrowth on the dead coral skeleton may stimulate further bleaching and eventually cause mortality in adjacent corals.

Figure 3. Reef degradation by coral-algae interactions. Progressing coral mortality by interaction with algae may act on different scales, ranging from (A) coral colony level to (B) community or ecosystem level. These interactions may involve algae-stimulated nitrogen fixation as a mechanism to interrupt the coral–algae symbiosis. Photo credits: N. Rädecker (A), M. Naumann (B).

**Future perspectives**

Although direct evidence is still scarce, the ubiquitous distribution of nitrogen cycling microbes in reef-building corals suggests a high functional relevance of this group for the holobiont. Future research directions should aim to increase our understanding of the possible implications of these symbiotic interactions within the holobiont in order to shed light on the underlying mechanisms of the responses of tropical reef-building corals to present and future anthropogenic changes (Box 2).
Recent technological advances in the biological sciences may help to answer these questions (for a detailed review see Pernice & Levy (88)). For instance, the development and application of culture-independent methods to characterize microbial communities has changed our understanding of the coral microbiome. In particular, the application of metatranscriptomics to disentangle expressed functions in different holobiont compartments (89) will help to decipher which microbial partners are important drivers of nitrogen cycling in corals. In this context, the growing number of available (meta-)genomes and (meta-)transcriptomes of corals, *Symbiodinium* and bacterial partners, will allow elucidation of key genes involved in nitrogen cycling and their distribution across holobiont compartments. For example, DMSP biosynthesis was only recently discovered in corals, a process that was believed to be restricted to marine algae and plants (90). It is thus important to test the established understanding of the distribution of genes related to nitrogen cycling between the coral host and its symbiotic partners through incorporation of data from multiple species and under different conditions.

Furthermore, the emerging use of stable isotope analysis and isotopic labeling provides an excellent tool to identify the fate of metabolic products in corals (47, 48, 91). In combination with NanoSIMS technology (27, 29, 53, 92), such approaches will enable us to understand how nitrogen uptake and nitrogen fixation affects the various symbiotic partners within the coral holobiont. However, due to the technical limitations from rinsing and dehydration steps during sample preparation, NanoSIMS measurements appear less suitable for detection of nitrification or denitrification. These new technological advances are most valuable when integrated into classical ecological approaches such as the indirect measurement of nitrogen fixation rates via the acetylene reduction assay (93). Such integrated applications will allow for the holistic understanding of nitrogen cycling in the coral holobiont, which is urgently needed to address the consequences of anthropogenically-driven environmental change in coral reefs.

**Box 2. Outstanding questions**

- What are the main functional players of the holobiont microbial community involved in coral-associated nitrogen cycling pathways, and how are shifts in diversity and abundance of these functional groups related to changes in the nitrogen cycling capacity of corals?
- How is the coral host involved in nitrogen cycling and exerting control over microbial nitrogen cycling activity?
- To what extent can corals adjust to anthropogenic changes, such as eutrophication, by altering their nitrogen cycling capacity?
- How is coral-associated nitrogen cycling governed by environmental conditions, and what are the implications for coral bleaching and disease?
Concluding remarks

The status of coral health is traditionally largely interpreted as the consequence of the interactions of corals and their endosymbiotic dinoflagellates. With an emerging characterization of the structure and function of the coral microbiome, this simplification may no longer be adequate. Rather, we are provided with a more holistic understanding of functional partitioning within the coral holobiont and the importance of associated microbes. Particularly microbial nitrogen cycling may play a crucial role in stabilizing or destabilizing the holobiont assemblage and function depending on environmental conditions. Shifts in the nitrogen cycling capacity may provide corals with a potential mechanism to persist in variable environments, such as those occurring through anthropogenic eutrophication. At the same time, stressors such as global warming and DOC enrichment may alter interactions of coral hosts and diazotrophs from a mutualistic to an opportunistic pathogenic association. Loss of control over this important microbial functional group by the host may have important consequences for the stability of the coral–algae symbiosis. In order to address this further, the mechanisms by which coral-associated microbes are involved in coral bleaching or diseases remain yet to be investigated.

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Appendix


ERKLÄRUNG

Bremen, den 26 Juli 2016

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

Coral holobiont functioning under global environmental change

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

______________________________
Claudia Pogoreutz