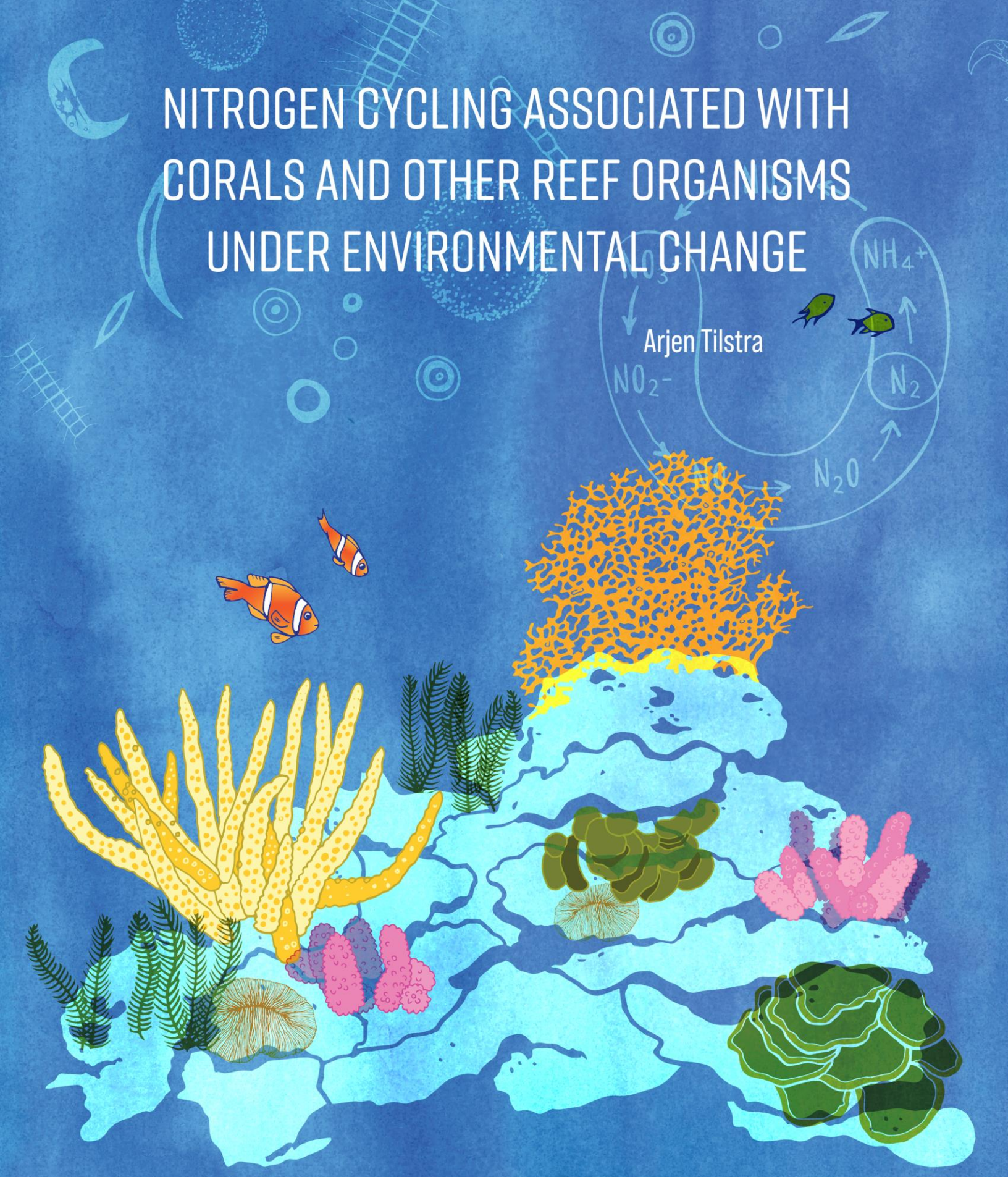
NITROGEN CYCLING ASSOCIATED WITH CORALS AND OTHER REEF ORGANISMS UNDER ENVIRONMENTAL CHANGE

Arjen Tilstra



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Colloquium: July 30th 2020, 2pm

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This thesis is dedicated to the memory of my loving mother,
Marrigje Tilstra-Post

- voor altijd in ons hart -

Nitrogen cycling associated with corals and other reef organisms under environmental change

Doctoral Thesis

for the attainment of the academic degree of

Doktor der Naturwissenschaften
- Dr. rer. nat. -

submitted to the Department of Biology and Chemistry
at the University of Bremen

in June 2020

Supervised by Prof. Dr. Christian Wild

The present work was carried out from August 2016 to March 2020 at the Center for Environmental Research and Sustainable Technology (UFT), University of Bremen, Germany

This work was financed by the German Research Foundation (DFG; Wi 2677/9-1) and baseline funds from the King Abdullah University of Science and Technology in Saudi Arabia

Summary

Nitrogen (N) is a limiting nutrient in highly productive tropical coral reefs, despite its key role for primary production. This requires efficient (re)cycling of N by the dwelling organisms, including the key reef ecosystem engineers, the hard corals. As such, corals evolved symbiotic relationships with eukaryotic and prokaryotic microbes, together called a holobiont, which aid in nutrient acquisition and recycling. The nutrient exchange symbiosis between the coral host and the eukaryotic photosynthetic dinoflagellates of the family Symbiodiniaceae has given corals an ecological advantage over other functional groups such as algae. The Symbiodiniaceae provide the coral host with carbon (C) rich photosynthates, while in return, the Symbiodiniaceae receive N and phosphorus (P). Additionally, diazotrophs, microbes capable of fixing atmospheric dinitrogen (N_2), can provide the coral holobiont with bioavailable N. Coral holobionts benefit from low internal availability of N as N-limitation may maintain steady translocation of the photosynthates on which the corals rely. Thus, coral holobionts may be particularly susceptible to increases in (environmental) dissolved inorganic N (DIN) due to e.g. anthropogenic input, or stimulated activity of diazotrophs. As such, corals likely have mechanisms in place for the alleviation of excess N, i.e. denitrification, which may ultimately aid coral functioning.

This thesis aims at extending the current knowledge on biogeochemical cycling of N associated with coral holobionts. Specifically, in addition to N_2 fixation, we tested whether the antagonistic N-cycling pathway to N_2 fixation, i.e. denitrification, is an active pathway in coral holobionts and whether it is affected by environmental change. In addition, we measured N-cycling pathways associated with other coral reef organisms and substrates under environmental change. This allowed us to make inferences for coral reef functioning when exposed to global and local stressors. We applied a combination of physiological and molecular analyses and used the strong seasonality of the northern and central Red Sea as a natural laboratory.

Our findings reveal that denitrification was actively associated with all investigated coral species. Similar to diazotrophy, denitrification may thus be ubiquitously associated with coral holobionts. Under stable environmental conditions, denitrification and N_2 fixation aligned and both N-cycling pathways correlated with Symbiodiniaceae cell densities. Thus, the relationship between denitrification and N_2 fixation may be the result of a shared organic C limitation (by translocated photosynthates from the Symbiodiniaceae) within the holobiont. Higher seasonal availability of DIN (leading to higher DIN:dissolved inorganic P [DIP] ratios) dynamically shifted the ratio of denitrifiers and diazotrophs, in favour of the denitrifiers. The proliferation of Symbiodiniaceae suggests incomplete alleviation of excess N by denitrification. Indeed, Symbiodiniaceae cell densities also correlated with environmental DIN availability. In response to moderate *in situ* eutrophication of DIN and DIP, both N-cycling pathways more than doubled in activity. Surprisingly, the Symbiodiniaceae populations remained stable. In addition, there was no significant incorporation of N originating from the eutrophication event in the Symbiodiniaceae. This suggests that N-limitation was maintained, likely assisted by denitrification. These findings suggest that the dynamic interplay of denitrification and N_2 fixation may regulate Symbiodiniaceae populations, but the extent to which they maintain N-limitation may depend on the environmental availability of DIN and DIP.

By comparing coral holobiont associated N-cycling to other functional groups on coral reefs, we postulate that under local and global stress scenarios, coral holobionts may lose the competition for space to algae as they 1) can strongly capitalize on (anthropogenic) nutrient inputs, 2) have high associated N_2 fixation

rates that increase in response to ocean warming and moderate N/P eutrophication, and/or 3) have low associated denitrification. Turf algae and coral rubble exhibited ~100-fold higher N_2 fixation rates compared to hard corals. Contrastingly, denitrification rates were as low as those associated with hard corals. Therefore, coral reefs in the process of shifting towards algae dominance may get caught in a positive feedback loop where dead coral (coral rubble) is rapidly overgrown by algae which in return naturally provide the reef with bioavailable N. This may facilitate higher growth rates of reef algae.

Collectively, the results described in this thesis suggest that the interplay of N_2 fixation and denitrification associated with coral holobionts may indeed aid in coral functioning by maintaining healthy populations of Symbiodiniaceae. Increased activity of diazotrophs induced by thermal stress, both associated with the coral holobiont and other dwelling organisms, as well as eutrophication of N may ultimately shift the coral holobionts' internal N:P ratios towards P limitation as denitrifiers may be unable to alleviate excess N. Thus, future management efforts should focus strongly on the local prevention of N eutrophication and the mitigation of global warming.

Zusammenfassung

Stickstoff (N) ist trotz seiner Schlüsselrolle für die Primärproduktion ein limitierender Nährstoff in hochproduktiven tropischen Korallenriffen. Dies erfordert effiziente Nutzung sowie Recycling von N durch die lebenden Organismen, einschließlich der wichtigsten Riffökosystemingenieure, der Hartkorallen. Deswegen entwickelten Korallen symbiotische Beziehungen zu eukaryotischen und prokaryotischen Mikroben, die zusammen als Holobiont bezeichnet werden und die Nährstoffaufnahme und das Recycling unterstützen. Die Nährstoffaustauschsymbiose zwischen dem Korallenwirt und den eukaryotischen, photosynthetischen Dinoflagellaten der Familie Symbiodiniaceae, hat Korallen einen ökologischen Vorteil gegenüber anderen funktionellen Gruppen wie Algen verschafft. Die Symbiodiniaceae versorgen den Korallenwirt mit kohlenstoffreichen Photosynthaten, während die Symbiodiniaceae im Gegenzug N und Phosphor (P) erhalten. Zusätzlich können Diazotrophe, Mikroben die in der Lage sind atmosphärischen Stickstoff (N_2) zu fixieren, den Korallenholobionten mit bioverfügbarem N versorgen. Korallenholobionten profitieren von einer geringen internen Verfügbarkeit von N, da die N-Begrenzung eine stetige Translokation der Photosynthaten aufrechterhalten kann, auf die sich die Korallen stützen. Somit können Korallenholobionten besonders anfällig für Erhöhungen von gelöstem anorganischem N (DIN) zum Beispiel durch anthropogenem Eintrag oder stimulierter Aktivität von Diazotrophen sein. Daher haben Korallen wahrscheinlich Mechanismen zur Reduzierung von überschüssigem N, das heißt Denitrifikation, die die Korallenfunktion unterstützen können.

Diese Arbeit zielt darauf ab, das aktuelle Wissen über den biogeochemischen Kreislauf von N in Verbindung mit Korallenholobionten zu erweitern. Insbesondere haben wir zusätzlich zur N_2-Fixierung getestet, ob der antagonistische Weg im N-Kreislauf zur N_2-Fixierung, Denitrifikation, ein aktiver Weg in Korallenholobionten ist und ob er durch Umweltveränderungen beeinflusst wird. Darüber hinaus haben wir Wege im N-Kreislauf gemessen, die mit anderen Korallenrifforganismen und -substraten assoziiert sind und von Umweltveränderungen beinflusst werden. Dies ermöglichte es uns, Rückschlüsse auf die Funktionen von Korallenriffen zu ziehen, wenn sie globalen und lokalen Stressfaktoren ausgesetzt sind. Wir haben eine Kombination aus physiologischen und molekularen Analysen angewendet und die starke Saisonalität des nördlichen und zentralen Roten Meeres als natürliches Labor genutzt.

Unsere Ergebnisse zeigen, dass die Denitrifikation mit allen untersuchten Korallenarten aktiv assoziiert war. Ähnlich wie bei der Diazotrophie kann die Denitrifikation daher allgegenwärtig mit Korallenholobionten assoziiert sein. Unter stabilen Umweltbedingungen stimmten Denitrifikation und N_2-Fixierung überein und beide N-Zykluswege korrelierten mit den Zelldichten von Symbiodiniaceae. Somit kann die Beziehung zwischen Denitrifikation und N_2-Fixierung das Ergebnis einer gemeinsamen organischen Kohlenstoffbegrenzung (durch translozierte Photosynthate aus den Symbiodiniaceae) innerhalb des Holobionten sein. Eine höhere saisonale Verfügbarkeit von DIN (was zu höheren DIN: gelöstem anorganischem P (DIP)-Verhältnissen führte) verschob das Verhältnis von Denitrifikatoren und Diazotrophen dynamisch zugunsten der Denitrifikatoren. Die Proliferation von Symbiodiniaceae deutet auf eine unvollständigen Abbau des überschüssigen N durch Denitrifikation hin. Tatsächlich korrelierten die Zelldichten von Symbiodiniaceae auch mit der Verfügbarkeit von DIN in der Umwelt. In Reaktion auf eine moderate *in situ* Eutrophierung von DIN und DIP verdoppelten sich beide N-Zykluswege in ihrer Aktivität. Überraschenderweise blieben die Symbiodiniaceae-Populationen stabil. Darüber hinaus gab es keinen signifikanten Einbau von N, das aus dem Eutrophierungsereignis stammte, in den Symbiodiniaceae. Dies

legt nahe, dass die N-Begrenzung erhalten wurde, wahrscheinlich unterstützt durch Denitrifikation. Diese Ergebnisse legen nahe, dass das dynamische Zusammenspiel von Denitrifikation und N_2-Fixierung die Populationen von Symbiodiniaceae regulieren kann, das Ausmaß, in dem sie die N-Begrenzung erhalten, jedoch von der Verfügbarkeit von DIN und DIP aus der Umwelt abhängen kann.

Durch den Vergleich des mit Korallenholobionten assoziierten N-Zyklus mit anderen funktionellen Gruppen in Korallenriffen postulieren wir, dass Korallenholobionten unter lokalen und globalen Stressszenarien die Konkurrenz um Platz mit Algen verlieren können: 1) da diese stark von (anthropogenen) Nährstoffeinträgen profitieren können, 2) hohe N_2-Fixierungsraten haben die als Reaktion auf die Erwärmung des Ozeans und eine moderate N / P-Eutrophierung zunehmen und / oder 3) eine geringe assoziierte Denitrifikation aufweisen. Aufwuchsalgen und Korallengestein zeigten im Vergleich zu Hartkorallen eine 100-fach höhere N_2-Fixierungsrate. Im Gegensatz dazu waren die Denitrifikationsraten so niedrig wie bei Hartkorallen. Daher können Korallenriffe, die sich in Richtung Algen-Dominanz verlagern, in eine positive Rückkopplungsschleife geraten, in der tote Korallen (Korallengestein) schnell von Algen überwachsen werden, die das Riff auf natürliche Weise mit bioverfügbarem N versorgen. Dies kann höhere Wachstumsraten von Riffalgen ermöglichen.

Zusammengenommen legen die in dieser Arbeit beschriebenen Ergebnisse nahe, dass das Zusammenspiel von N_2-Fixierung und Denitrifikation in Verbindung mit Korallenholobionten tatsächlich die Korallenfunktion unterstützen kann, indem gesunde Populationen von Symbiodiniaceae erhalten bleiben. Eine erhöhte Aktivität von Diazotrophen, die durch thermischen Stress induziert werden und sowohl mit dem Korallenholobionten als auch mit anderen lebenden Organismen assoziiert sind, sowie die Eutrophierung von N können letztendlich die internen N:P-Verhältnisse der Korallenholobionten in Richtung P-Begrenzung verschieben, da Denitrifikatoren möglicherweise nicht in der Lage sind, überschüssiges N abzubauen. Zukünftige Managementbemühungen sollten sich daher stark auf die lokale Prävention der N-Eutrophierung und die Eindämmung der globalen Erwärmung konzentrieren.

Acknowledgements

Despite the blood, sweat and tears I poured into writing this thesis, no section has been as difficult for me to write as this one. Not just because I don't want to miss anyone, but because one very important person in my life won't be able to read it.

My fascination for all things living and the respect I have of our beautiful planet has been inspired by my parents. We watched many nature documentaries together and I fondly remember watching Waku Waku (animal-facts game show) together on national tv as a kid. But it was my mother who took me to see Jurassic Park when I was 9 years old. This movie would inspire me to pursue a career in science. I wanted to be the scientist that took the eggshell of the baby velociraptors head. It's safe to say I would not have become a scientist if it wasn't for my mother. In February 2019 she was diagnosed with a disease that has caused so many of us so much pain and sadness. My mother lost her battle on April 19th 2019, on Good Friday. Mams, er gaat geen dag voorbij dat ik niet aan je denk, dank je voor alles wat je voor me hebt betekent in mijn leven, ik mis je en hou van je. During my mother's sickbed, my father gave my mother the best and most loving care she could wish for to which I'm extremely grateful. While the pain of loss remains, it becomes more bearable with time. I hope to share many more adventures and whiskies with you in the future, dad. Ik hou van je, paps.

Of course, this PhD would not have been possible without Prof. Dr. Christian Wild. I thank you sincerely for picking me out of all applicants now over 4 years ago. Thank you for guiding me every step of the way, in good times and bad times. I am looking forward to working together more in the near future. I'm also looking forward to more delicious Obatzda at our lab events!

I also want to thank Prof Dr. Christian Voolstra, for allowing me to come to KAUST, letting me do what I wanted to do, and guidance in pursuing this PhD. I also want to thank ex-KAUSTians Claudia Pogoreutz, Nils Rädecker and Florian Roth for their patience and extremely valuable help during the last 4 years. This thesis would not have been possible without all of you. I want to thank Holger Anlauf for taking me to my first Red Sea snorkelling trip allowing me to see a coral reef for the first time in my life (I know, I'm still amazed it took this long!). I also want to thank Hagen Gegner and Saeed Salem Bunna for making KAUST a truly unforgettable experience. Finally, I thank everyone in the Reef Genomics group for making me feel welcome, you truly are a special bunch in every positive sense of the word.

I want to express my thanks to all the friends I made in Bremen, many of which are also colleagues, for making the last 4 years so awesome: Sophie, Carmen, Stefan, Simone, Ale, Stefanos, my fellow whisky afficionados Filip and Tobi, Valentina, Kathrin, Lissy, Nan, Chrissy, Denis (I am sorry, mavidori), Nauras, and my office mates Ameris, Anna, Susana and my fellow nitrogen cycling geek Herr El-Khaled. You all are the loveliest people imaginable.

To all my friends and family in the Netherlands, I love you all and I miss you!

Thanks to my cousin Monique for designing the front cover and related illustrations.

A special thank you goes to Roy and Nicolien and their two beautiful kids which I may proudly call myself the godfather of. Thank you for always being there for me.

Thank you to all committee members, and Bert Hoeksema for reviewing this thesis.

I want to thank people who have inspired me in one way or another: Tim Wijgerde, Tanne Hoff, Ronald Osinga, Joana Falcao Salles, Ed Schreuter, Jeanine Olsen, and Francisco Dini-Andreote. I hope we see each other again in the future!

We thank the Bremen University library for their Open Access policy which allowed us to publish four from the six published chapters in Open Access journals.

I'd like to thank everyone from the Marine Ecology and CORE group, and those people involved in the NICE project and ScienceChat that I may have forgotten to mention by name.

Lastly, I want to thank my beloved Moira, for being there for me when I needed you most, for listening to what can often only be described as nonsense, for bringing me back up when I was doubting myself. Thank you for everything. We drive it like we stole it, I love you.

Table of contents

List of abbreviations and symbols

2D	Two-dimensional
3D	Three-dimensional
ΔCt	delta Cycle threshold
%	percent
°C	degrees Celsius
α	alpha
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
µmol	micromol
Ah	*Acropora hemprichii*
A.K.A.	Also Known As
ANOVA	Analysis of variance
ANOSIM	Analysis of similarity
Ap	*Acropora pharaonis*
BIOENV	Biota-environmental matching
C_2H_2	acetylene
C_2H_4	ethylene
C	Carbon
Ce	*Ctenactis echinata*
cf.	*confer/conferatur*
chl *a*	chlorophyll *a*
cm	centimeter
CMOR	Coastal & Marine Resources Core Lab Services
COBRA	Combined Acetylene Blockage and Reduction Assay
C_{org}	Organic carbon
CPCe	Coral Point Count with Excel extension
CR	Community Respiration
Ct	Cycle threshold
CTD	Conductivity–Temperature–Depth
d	day
DAAD	Deutscher Akademischer Austauschdienst
df	degrees of freedom
DFG	Deutsche Forschungsgemeinschaft
DHC	denitrification-heterotrophic capacity (hypothesis)
DIC	Dissolved Inorganic Carbon
DIN	Dissolved Inorganic Nitrogen
DIN:DIP	ratio of DIN and DIP

DIN:PO_4^{3-}	ratio of DIN to PO_4^{3-}
DIP	Dissolved Inorganic Phosphorus
DistLM	distance-based linear model
DNA	DeoxyriboNucleic Acid
DOC	Dissolved Organic Carbon
e.g.	*exempli gratia*
E	Efficiency
Ef	*Echinopora fruticulosa*
et al.	*et alia*
GPP	Gross Primary Production
h	hour
HCl	hydrochloric acid
HDPE	High Density Poly-Ethylene
i.e.	*id est*
ITS2	Internal Transcribed Spacer 2
KAUST	King Abdullah University of Science and Technology
km	kilometer
L	Liter
LOQ	Limits of Quantification
m	meter
Md	*Millepora dichotoma*
mg	milligram
Milli-Q	Ultrapure water
Mio	million/millions
mL	milliliter
mm	millimeter
mmol	millimol
MSS	Marine Science Station
N	Nitrogen
n	sample size
N_2	dinitrogen
N_2O	nitrous oxide
NaCl	Sodium Chloride
nar	nitrate reductase
NCP	Net Community Production
ng	nanogram
NH_4^+	ammonium
nir	nitrite reductase
nmol	nanomol
NO	nitric oxide
NO_x	Nitrogen Oxides
NO_2^-	nitrite

NO_3^-	nitrate
nor	nitric oxide reductase
nos	nitrous oxide reductase
N:P	ratio of N to P
ns	not significant
nsa	no specific amplification
O_2	oxygen
OPA	ortho-phthalaldidehyde solution
P	Phosphorus
p	*p* value
PAR	Photosynthetically Active Radiation
PCO	principal coordinate analysis
PCR	Polymerase Chain Reaction
PERMANOVA	permutational multivariate analysis of variance
Pg	*Pleuractis granulosa*
P_g	gross photosynthesis
Pl	*Porites lutea*
PN	Particulate Nitrogen
P_n	net photosynthesis
PO_4^{3-}	phosphate
POC	Particulate Organic Carbon
POM	Particulate Organic Matter
ppb	parts per billion
ppm	parts per million
PSU	Practical Salinity Unit
Pv	*Pocillopora verrucosa*
qPCR	quantitative Polymerase Chain Reaction
r	correlation coefficient
r^2	coefficient of determination
R	dark respiration
rcf	relative centrifugal force
rpm	rounds per minute
R_{ref}	Reference ratio
rRNA	ribosomal RiboNucleic Acid
s	second
SCUBA	Self-Contained Underwater Breathing Apparatus
SD	Standard Deviation
SE	Standard Error
Sp	*Stylophora pistillata*
sp.	species
spp.	*species pluralis*
temp	temperature

TOC	Total Organic Carbon
W	coefficient of concordance
Xe	*Xenia* sp.
yr	year
zoox.	Zooxanthellae

List of publications and manuscripts included in this thesis

Tilstra A, Bednarz VN, Cardini U, van Hoytema N, Al-Rshaidat MMD, Wild C (2017) Seasonality affects dinitrogen fixation associated with two common macroalgae from a coral reef in the Northern Red Sea. *Marine Ecology Progress Series* 575:69-80 https://doi.org/10.3354/meps12206

Tilstra A, van Hoytema N, Cardini N, Bednarz VN, Rix L, Naumann MS, Al-Horani FA, Wild C (2018) Effects of water column mixing and stratification on planktonic primary production and dinitrogen fixation on a Northern Red Sea coral reef. *Frontiers in Microbiology* 9:2351 https://doi.org/10.3389/fmicb.2018.02351

Tilstra A, Pogoreutz C, Rädecker N, Ziegler M, Wild C, Voolstra CR (2019) Relative diazotroph abundance in symbiotic Red Sea corals decreases with water depth. *Frontiers in Marine Science* 6:372 https://doi.org/10.3389/fmars.2019.00372

Tilstra A, El-Khaled YC, Roth F, Rädecker N, Pogoreutz C, Voolstra CR, Wild C (2019) Denitrification aligns with N_2 fixation in Red Sea corals. *Scientific Reports* 9:19460 https://doi.org/10.1038/s41598-019-55408-z

El-Khaled YC, Roth F, **Tilstra A**, Rädecker N, Karcher DB, Kürten B, Jones BH, Voolstra CR, Wild C (accepted) *In situ* eutrophication stimulates dinitrogen fixation and denitrification in key functional groups of Red Sea coral reefs. *Marine Ecology Progress Series* https://doi.org/10.3354/meps13352

Karcher DB, Roth F, Carvalho S, El-Khaled YC, **Tilstra A**, Kürten B, Struck U, Jones BH, Wild C (2020) Nitrogen eutrophication particularly promotes turf algae in coral reefs of the central Red Sea. *PeerJ* 8:e8737 https://doi.org/10.7717/peerj.8737

List of manuscripts in preparation for submission included in this thesis

Tilstra A, Roth F, El-Khaled YC, Pogoreutz C, Rädecker N, Voolstra CR, Wild C (in prep.) Evidence for dynamic environmental control of coral holobiont nitrogen cycling. *Royal Society Open Science*

El-Khaled YC, Roth F, Rädecker N, **Tilstra A**, Karcher DB, Kürten B, Jones BH, Voolstra CR, Wild C (in prep.) Prospective phase shifts from corals to algae domination promote nitrogen accumulation in reefs. *Ecosystems*

Declaration on the contribution of the candidate to a multi-author article/manuscript which is included as a chapter in the submitted doctoral thesis

Contribution of the candidate is given in % of the total work load (up to 100% for each category)

Chapter 2 | **Effects of water column mixing and stratification on planktonic primary production and dinitrogen fixation on a northern Red Sea coral reef**

Experimental concept and design	0 %
Experimental work and/or acquisition of (experimental) data	0 %
Data analysis and interpretation	80 %
Preparation of figures and tables	100 %
Drafting of the manuscript	50 %

Chapter 3 | **Seasonality affects dinitrogen fixation associated with two common macroalgae from a coral reef in the northern Red Sea**

Experimental concept and design	0 %
Experimental work and/or acquisition of (experimental) data	0 %
Data analysis and interpretation	80 %
Preparation of figures and tables	100 %
Drafting of the manuscript	100 %

Chapter 4 | **Relative diazotroph abundance in symbiotic Red Sea corals decreases with water depth**

Experimental concept and design	0 %
Experimental work and/or acquisition of (experimental) data	0 %
Data analysis and interpretation	100 %
Preparation of figures and tables	100 %
Drafting of the manuscript	100 %

Chapter 5 | **Denitrification aligns with N_2 fixation in Red Sea corals**

Experimental concept and design	50 %
Experimental work and/or acquisition of (experimental) data	75 %
Data analysis and interpretation	90 %
Preparation of figures and tables	100 %
Drafting of the manuscript	95 %

Chapter 6 | **Denitrification primer assessment**

Experimental concept and design	100 %
Experimental work and/or acquisition of (experimental) data	100 %
Data analysis and interpretation	100 %
Preparation of figures and tables	100 %
Drafting of the manuscript	100 %

Chapter 7 | **Evidence for dynamic environmental control of coral holobiont nitrogen cycling**

Experimental concept and design	100 %
Experimental work and/or acquisition of (experimental) data	100 %
Data analysis and interpretation	80 %
Preparation of figures and tables	100 %
Drafting of the manuscript	100 %

Chapter 8 | ***In situ*** **eutrophication stimulates dinitrogen fixation, denitrification and productivity in Red Sea coral reefs**

Experimental concept and design	10 %
Experimental work and/or acquisition of (experimental) data	0 %
Data analysis and interpretation	20 %
Preparation of figures and tables	20 %
Drafting of the manuscript	5 %

Chapter 9 | **Nitrogen eutrophication particularly promotes turf algae in coral reefs of the central Red Sea**

Experimental concept and design	10 %
Experimental work and/or acquisition of (experimental) data	10 %
Data analysis and interpretation	15 %
Preparation of figures and tables	0 %
Drafting of the manuscript	5 %

Chapter 10 | **Prospective phase shifts from corals to algae domination promote nitrogen accumulation in reefs**

Experimental concept and design	10 %
Experimental work and/or acquisition of (experimental) data	0 %
Data analysis and interpretation	10 %
Preparation of figures and tables	20 %
Drafting of the manuscript	5 %

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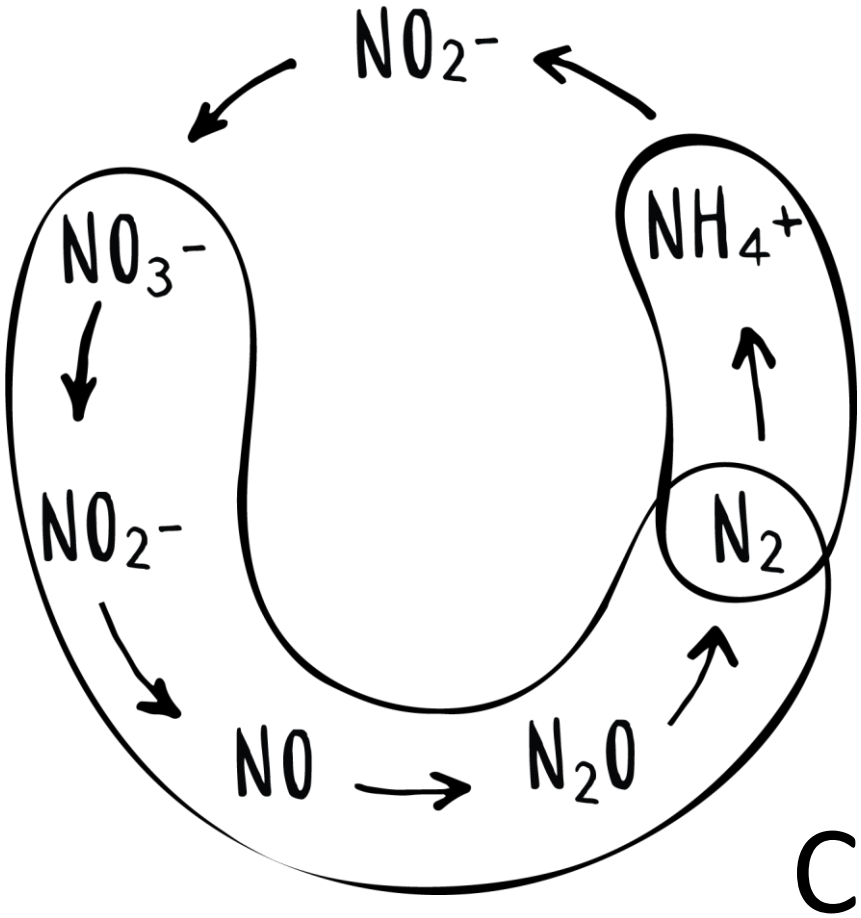

Chapter 1

Chapter 1 | **General introduction**

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Keywords: Coral reefs | holobiont | Symbiodiniaceae | nitrogen cycling | dinitrogen fixation | denitrification

1.1 | Nutrient exchange symbiosis in coral holobionts

Coral reefs are inhabited by thousands of often endemic species of many phyla, and are thus considered biodiversity hotspots[1–3]. Central to coral reefs are hard corals (Phylum: Cnidaria) – also known as stony, reef-building, scleractinian or hermatypic corals – as they build skeletons made from calcium carbonate (aragonite). As such, hard corals act as ecosystem engineers[4], as they form the limestone foundation and 3D structure of the benthos, which can act as a substrate or habitat for many other organisms[5], including fish, soft corals, gorgonians, sponges, and (macro)algae, among many others. Healthy coral reefs are thriving ecosystems inhabited by highly specialized organisms, as they inhabit environments of extreme nutrient scarcity (oligotrophy). However, despite this scarcity, coral reefs belong to the world's most productive ecosystems[6], a contradiction commonly known as the Darwin's Paradox. The oligotrophic nature of coral reefs demands efficient use and recycling of the available nutrients by the dwelling organisms[7,8], including the corals themselves. As such, corals evolved a highly biodiverse microbiome consisting of eukaryotic and prokaryotic microbes[9] with members that aid in nutrient provision and (re)cycling[10,11], among other traits. Together, the coral host and its microbiome are referred to as the coral holobiont.

The most heavily studied coral associated microbes are the photosynthetic dinoflagellates of the family Symbiodiniaceae[12] (colloquially known as zooxanthellae) that live in a mutualistic nutrient exchange symbiosis with the coral host. Symbiodiniaceae provide the coral host with carbon rich photosynthates[13,14]. In fact, up to 95 % of the corals' carbon demand can originate

Box 1 | Diazotrophy (N_2 fixation)

Dinitrogen (N_2) fixation is the biological fixation of atmospheric N_2 by a diverse range of Bacteria and Archaea, known as diazotrophs. Diazotrophs use atmospheric N_2 as a substrate and convert it (through the nitrogenase enzyme complex; encoded by the *nif* gene cluster) into bioavailable ammonium. The activity of the nitrogenase enzyme complex is highly affected by its environment, i.e. increased oxygen availability may deactivate the dinitrogenase reductase[98], one of the two subunits of the enzyme complex. Furthermore, the availability of bioavailable nitrogen may also hamper nitrogenase activity[71] through so called ammonia switch-off[99]. On the contrary, increasing water temperatures and irradiance may stimulate the enzymatic activity of nitrogenase[51,61,71,98]. Diazotrophy has been demonstrated to be associated with many coral reef substrates and organisms including soft corals[72], sponges[71], turf algae[71], macroalgae[77,78], coral rock[71], and silicate and carbonate sediments[100], among others. Furthermore, N_2 fixation was recognized as a ubiquitous nitrogen cycling pathway in hard coral skeletons as early as the late 70s[101], and later confirmed for living hard corals by Williams et al.[102] and Shashar et al.[103]. These studies, and many others, used the common method known as the Acetylene Reduction Assay which can also be used to quantify denitrification[104]. At present, N_2 fixation can also be inferred from labelled isotope techniques[80,105] and the diazotrophic potential of coral holobionts can be assessed by quantifying abundances of the functional marker gene *nifH*[82].

from translocation of photosynthates from the Symbiodiniaceae to the coral host[15]. In return, the Symbiodiniaceae acquire nutrients from the coral host, e.g. nitrogen and phosphorus, which can be obtained via heterotrophic feeding, by uptake from the water column, and/or internal (re)cycling[16,17]. In addition, prokaryotic dinitrogen (N_2) fixers, called diazotrophs (see Box 1 for more information), associated with corals may provide *de novo* bioavailable nitrogen in the form of ammonium, the preferred uptake nitrogen source by the Symbiodiniaceae[18–20]. The remaining organic carbon demand of the coral host can be obtained via heterotrophic feeding, rendering corals as inherent mixotrophs. In fact, heterotrophy can account for more than half of the metabolic carbon demand in healthy corals and may reach up to 100 % in heavily stressed corals[21,22].

1.2 | Coral reefs under global and local environmental change

Corals reefs currently face unprecedented pressure from anthropogenic (i.e. human induced) global stressors[23]. Anthropogenic climate change, as a result of global warming, has resulted in increased sea surface temperatures on coral reefs worldwide. As a result, sea surface temperatures may exceed summer thresholds more often[24], which may lead to coral bleaching (Box 2) and eventually death of the coral organism if the heating persists[25,26]. Additionally, overfishing, eutrophication, and damage from tourism[27–30] among others, contribute to coral reef stress as local threats. Together, these anthropogenic stressors have caused a decline in worldwide coral cover[31,32]. It is estimated that more than half of coral cover was lost during the 20th century[33,34]. Furthermore, aragonite accretion, i.e. calcification, mediated by hard corals is an energy-costly process[17]. As such, coral holobionts rely on the Symbiodiniaceae by translocating energy-rich photosynthates[35]. As a result, climate change can cause hard corals to lose their role as ecosystem engineers[36]. With the predicted increase of bleaching events in the upcoming future[37,38] and associated reductions in recovery time[39], not only may we lose one of the most aesthetically pleasing ecosystems[40], millions of people may likely lose their livelihoods because of the ecosystem services provided by coral reefs[41,42].

Box 2 | Coral bleaching

Prolonged exposure to stressors such as thermal anomalies[25,106], elevated solar irradiance[107,108], and (in)organic eutrophication[50,109,110] can cause the progressive dissociation of the symbiosis between the coral host and the Symbiodiniaceae, thereby promoting coral bleaching[111]. Coral bleaching is defined as the loss of the Symbiodiniaceae cells and/or their photopigments. This causes the coral to lose its dark colour and hence leads to the visibility of the coral skeleton through the coral tissue causing a white (hence bleached) phenotype. Widespread bleaching events have been recorded in recent decades[112–114] but have been particularly high in frequency in recent years. Australia's Great Barrier Reef for instance was hit especially hard by unprecedented back to back bleaching in response to El Nino events in 2016 and 2017[115,116], and is currently (2020) facing its 3rd major bleaching event since 2016[117].

While the specific underlying mechanism for coral bleaching remains still to be properly determined, one explanation is positing that coral bleaching is related to nutrient availability; in particular, the disruption of nitrogen limitation for the Symbiodiniaceae[43,44]. Thus, maintaining a balanced coral – Symbiodiniaceae symbiosis may require a relatively stable nitrogen limited environment for the Symbiodiniaceae and, as such, nitrogen cycling microbes may play a key role[43].

1.3 | The potential role of nitrogen cycling microbes

The intact symbiosis: A nitrogen limited state

Nitrogen is an essential macronutrient for coral holobiont productivity[45,46], but is ironically often the

limiting nutrient on coral reefs. However, the low nitrogen availability serves the coral holobionts own interest; limited nitrogen availability for Symbiodiniaceae will keep them from using the self-produced photosynthates for their own metabolism. Thus, maintaining *in hospite* nitrogen limitation is crucial for the coral – Symbiodiniaceae symbiosis. An interruption of nitrogen limitation can bring the Symbiodiniaceae in a phosphorus-limited state[43,44]. Wiedenmann et al.[47] demonstrated that this can cause the Symbiodiniaceae to replace phospholipids in the thylakoid membranes of the chloroplasts with sulpholipids. The latter weakens the stability of the thylakoid membranes making them more susceptible to e.g. oxidation, thus lowering the coral holobionts bleaching threshold[48]. Changes to the nitrogen:phosphorus stoichiometry were recently linked to the decline of coral reefs in the Caribbean[49]. More evidence in support of this was found by Pogoreutz et al.[50]. These authors described that the addition of dissolved organic carbon (DOC) to the incubation water of corals caused an increased activity of diazotrophs. This resulted in the increased availability of bioavailable nitrogen for the Symbiodiniaceae and a subsequent disruption of nitrogen limitation. As a result, the corals bleached despite a lack of warming or light stress. Thus, nitrogen is a key nutrient for understanding coral holobiont functioning[43].

The role of nitrogen cycling microbes when coping with fluctuations in nitrogen availability

The stability of coral reef ecosystems relies on maintaining limited availability of nitrogen[49]. However, coral reefs, and by proxy coral holobionts, are exposed to both seasonal and diel fluctuations of nitrogen availability[51,52]. Nitrogen cycling in the world's oceans is mainly controlled by marine microbes[53], in particular by diazotrophs and denitrifiers[54–56]. Indeed, coral holobiont productivity is positively affected by the influx of diazotroph derived nitrogen[43,57–59] when nitrogen availability is scarce[51]. By extension, N_2 fixation aids in maintaining coral reef ecosystem productivity[60]. Though, the functional stability of coral holobionts relies on maintaining limited availability of nitrogen[43,47]. Natural temperature fluctuations, as a result of seasonality or water depth, may be positively correlated with the N_2 fixation potential[51,61,62], thus increasing the coral holobionts' nitrogen availability at the maximum temperatures[63]. As such, it is tempting to speculate that denitrification (see Box 3 for more information) may be an active pathway in coral reefs and coral holobionts as it was hypothesized that they play a putative role in alleviating nitrogen availability to ultimately maintain a nitrogen limited state for Symbiodiniaceae[43]. Thus, the interplay of denitrification and N_2 fixation may play an important role in maintaining relatively stable levels of bioavailable nitrogen,

Box 3 | Denitrification

Denitrification is a nitrogen cycling pathway that reduces nitrate to atmospheric N_2[118,119] and can thus be considered antagonistic to N_2 fixation (Box 1). The denitrification pathway consists of four main enzymes, i.e. nitrate reductase (converting nitrate to nitrite; encoded by the gene cluster *nar*), nitrite reductase (converting nitrite to nitric oxide; encoded by the gene cluster *nir*), nitric oxide reductase (converting nitric oxide to nitrous oxide; encoded by the gene cluster *nor*), and nitrous oxide reductase (converting nitrous oxide to atmospheric N_2; encoded by the gene cluster *nos*) (see Figure 5.1). The denitrification pathway may be susceptible to several inorganic nutrients as previously found in systems such as soils and (marine) sediments. As the substrate for denitrifiers, nitrate enrichment may stimulate denitrifier activity[119], whereas phosphorus may both be positively[120] or negatively correlated[121] with denitrifier abundances, depending on the individual members of the denitrifying community[122]. As a result, denitrifier abundances may be correlated with dissolved inorganic nitrogen and phosphorus, i.e. DIN:DIP, ratio's[121]. Moreover, denitrifier abundances may be influenced by temperature[123,124] and dissolved organic carbon[125]. Only a limited number of studies has thus far assessed denitrification associated with coral reef substrates/organisms, such as biogenic rock[84,85], sediments[86–89,126,127], and hard corals[90–92]. Methods to measure denitrification rates or abundances include the COmbined acetylene Blockage and Reduction Assay also known as COBRA[104], labelled isotopes[128], and by the quantification of functional marker genes[84].

not just on the level of the coral holobiont but also on the ecosystem level.

At the same time, nitrogen eutrophication, the influx of otherwise unavailable nitrogen through e.g. agricultural fertiliser runoff and (un)controlled sewage dumping or even natural upwelling[64], may reshape coral reefs[65,66] potentially leading to phase-shifts from hard coral dominance towards e.g. (macro)algal dominance[67,68]. Anticipated growth of human population will likely increase the risk of these eutrophication events[69,70], while simultaneously having to act as a potential source of food and income. Furthermore, sea surface temperatures are expected to exceed coral temperature thresholds more often in the future[37]. As such, diazotrophic activity is expected to increase with it[61,62,71,72]. It is, as of yet, unknown how denitrification (if present) associated with most coral reef functional groups respond to changes in water temperature and availability of nutrients. Hard coral holobionts exposed to ocean warming, organic and inorganic eutrophication often bleach[50,73,74], possibly due to disrupted nitrogen limitation[49,50]. Thus, increased nitrogen availability may affect functioning of the coral holobiont, and coral reef functioning by proxy[75], if not compensated for by denitrification activity. It is thus of great importance to understand the effects of abiotic variables on biogeochemical cycling of nitrogen on coral reefs[76] and hard coral holobionts in particular.

1.4 | Specific knowledge gaps

A holistic understanding of biogeochemical cycles on coral reefs requires knowledge on all associated functional groups. As such, there has been a lack of studies assessing the effects of environmental change on macroalgae associated N_2 fixation. Furthermore, to our knowledge there are no studies that related environmental change with N_2 fixation associated with planktonic communities directly overlaying a coral reef. Both macroalgae and phytoplankton may facilitate the influx of bioavailable nitrogen through N_2 fixation[77–79] but it is unknown if the diazotrophic communities associated with these functional groups responds similarly to environmental change as those diazotrophs associated with e.g. hard corals.

While coral holobiont associated N_2 fixation has received much attention in recent years[51,61,80–83], denitrification remains a critically understudied biogeochemical pathway in coral holobionts and on coral reef ecosystems despite the potential importance. While present on coral reefs (Box 3), denitrification rates have only been measured in biogenic rock[84,85] and sediments[86–89]. While also present in coral holobionts[90–92], studies assessing patterns of denitrification rates in coral holobionts are currently unavailable. Yang et al.[90] was the first study on coral holobionts that assessed the denitrifying community structure and its phylogenetic diversity. However, this study was done on one fully heterotrophic soft (i.e. non-reef-building) coral and one fully heterotrophic hard coral. As both denitrifiers and diazotrophs are mainly heterotrophic[93–95], the photosynthates translocated by the Symbiodiniaceae may provide their primary source of organic carbon[96]. As such, it is important to consider the potential interplay of denitrifiers, diazotrophs and Symbiodiniaceae on hard corals.

Many studies on nitrogen cycling in coral reefs often focus on a single pathway or focus on pathways that have a comparable function[89,90,92,97], i.e. the reduction of bioavailable nitrogen to N_2 by coupled nitrification and denitrification or from anaerobic ammonium oxidation (anammox). Only rarely are antagonistic pathways, i.e. N_2 fixation and denitrification, assessed for coral reef functional groups[88].

However, the interplay of these pathways may be key to coral reef ecosystem functioning[75]. It is thus of paramount importance to integrate both pathways in coral reef studies assessing biogeochemical nitrogen cycling.

1.5 | Aims and approach

Aims

Nitrogen is of prime importance for the functioning of coral reef organisms. Therefore, it is of profound importance to study biogeochemical processes such as nitrogen cycling under ambient and/or stressed conditions to get a more holistic understanding of coral reef ecosystems and by proxy coral reef functioning. Thus, in this thesis we aimed at assessing the effects of environmental change (ambient; due to seasonality or differences in water depth) and eutrophication (stressed) on nitrogen cycling associated with a range of coral reef associated functional groups, including hard corals.

We specifically aimed at answering the following research questions:

1) Is N_2 fixation associated with coral reef organisms affected by environmental change and how do these potential changes compare between organisms? What are the ecological implications?
2) Is denitrification an active pathway associated with coral holobionts? If yes, how does it align with abiotic and/or other coral holobiont associated biotic variables?
3) How are nitrogen cycling pathways associated with several functional groups affected by eutrophication? Do these functional groups incorporate the extra bioavailable nitrogen?
4) Which functional groups are the major contributors to nitrogen cycling on coral reefs? Do differences in benthos composition affect the cycling of nitrogen and what are the ecological implications?

Approach

All experiments were performed in the central and northern part of the oligotrophic Red Sea. The strong seasonality in both parts of the Red Sea was used as a natural laboratory to study the effects of abiotic variables such as temperature, light intensity, and (in)organic nutrient availability on functional group associated nitrogen cycling processes. Our approach included the molecular quantification of denitrifiers and/or diazotrophs, the physiological quantification of N_2 fixation and denitrification using acetylene assays, quantification of carbon and nitrogen content through elemental and stable isotope analysis, measurements of additional biotic variables such as oxygen fluxes (carbon fixation) and Symbiodiniaceae cell densities/mitotic indices, and monitoring of environmental parameters (abiotic variables) including water temperature, water depth, light intensity, salinity, pH, chlorophyll *a*, and (in)organic nutrients.

1.6 | Thesis structure and outline

This thesis is composed of 11 chapters. The first and last chapter act as a general Introduction and Discussion, respectively. The nine chapters in between consist of published manuscripts and manuscripts intended for publication and examine the effects of environmental change, both natural and anthropogenic, on functional group associated nitrogen cycling, i.e. diazotrophy (N_2 fixation) and denitrification (Figure 1.1). More specifically, these nine chapters are separated into four sections (based on the research questions), as follows:

Section 1: N_2 fixation associated with coral reef organisms in response to environmental change

In Chapter 2 we investigate the effects of seasonality on N_2 fixation associated with pelagic phytoplankton from a water column overlaying a coral reef. In this chapter we use an approach of physiological measurements of N_2 fixation. Additionally, biotic and abiotic variables were measured/monitored to assess potential relationships with N_2 fixation. In Chapter 3 we investigate the effects of seasonality on N_2 fixation associated with two macroalgae. Here we used a similar approach as the previous chapter. In both chapters we also compare N_2 fixation measured during that particular study with those found in parallel investigated organisms and substrates to assess potential behavioural differences between pelagic and all investigated benthic diazotrophs. Chapter 4 changes the approach by measuring N_2 fixation molecularly. In this chapter we measure relative *nifH* gene copy numbers, as a proxy for N_2 fixation, associated with the tissues of two hard corals along a depth gradient to assess whether shallow corals rely more on diazotrophy compared to their deeper conspecifics. Abiotic variables along this depth gradient were monitored and potential relationships with relative *nifH* gene copy numbers were assessed.

Section 2: Denitrification and N_2 fixation associated with coral holobionts

In Chapter 5 we assess denitrification associated with three coral species both molecularly and physiologically. To this end, a range of primers for all functional marker genes (see Figure 5.1) associated with the denitrification pathway were first tested on nine common Red Sea calcifying corals (Chapter 6). To assess whether molecularly obtained results could be used as a proxy for actual denitrification rates, a newly described physiological method was used to measure denitrification and N_2 fixation rates (i.e. combined blockage/reduction acetylene assay, A.K.A. COBRA). Finally, other coral associated biotic variables were measured to assess potential relationships with both denitrification and N_2 fixation rates. Then, in Chapter 7, we molecularly assess denitrification, in relation to diazotrophy, associated with two Red Sea hard corals in a seasonal resolution. Abiotic and biotic variables were monitored within each season to provide an environmental and biological context for observed seasonal patterns.

Section 3: Denitrification and N_2 fixation associated with coral reef functional groups in response to anthropogenic eutrophication

In Chapter 8 we investigate the effects of *in situ* eutrophication on N_2 fixation and denitrification rates associated with three functional groups, i.e. sediments, turf algae and hard coral. To this end we used COBRA to measure nitrogen cycling pathways. In addition, we measure oxygen fluxes to assess potential relationships with nitrogen cycling pathways. Chapter 9 follows the same *in situ* eutrophication experiment. However, in this chapter we examine the carbon and nitrogen content of above-mentioned functional groups + soft corals using elemental and stable isotope analyses to assess whether the nitrogen additions to the reef were incorporated in the functional group. Additionally, this chapter measured Symbiodiniaceae cell densities and mitotic indices of the involved hard corals.

Section 4: Denitrification and N_2 fixation at the coral reef ecosystem level

Chapter 10 investigates which functional groups are major contributors to nitrogen cycling to assess the potential effects on coral reef nitrogen cycling in case of prospective phase shifts from coral to algae dominance. COBRA was used to provide a relative budget for bioavailable nitrogen brought into the system via N_2 fixation and bioavailable nitrogen relieved from the system via denitrification.

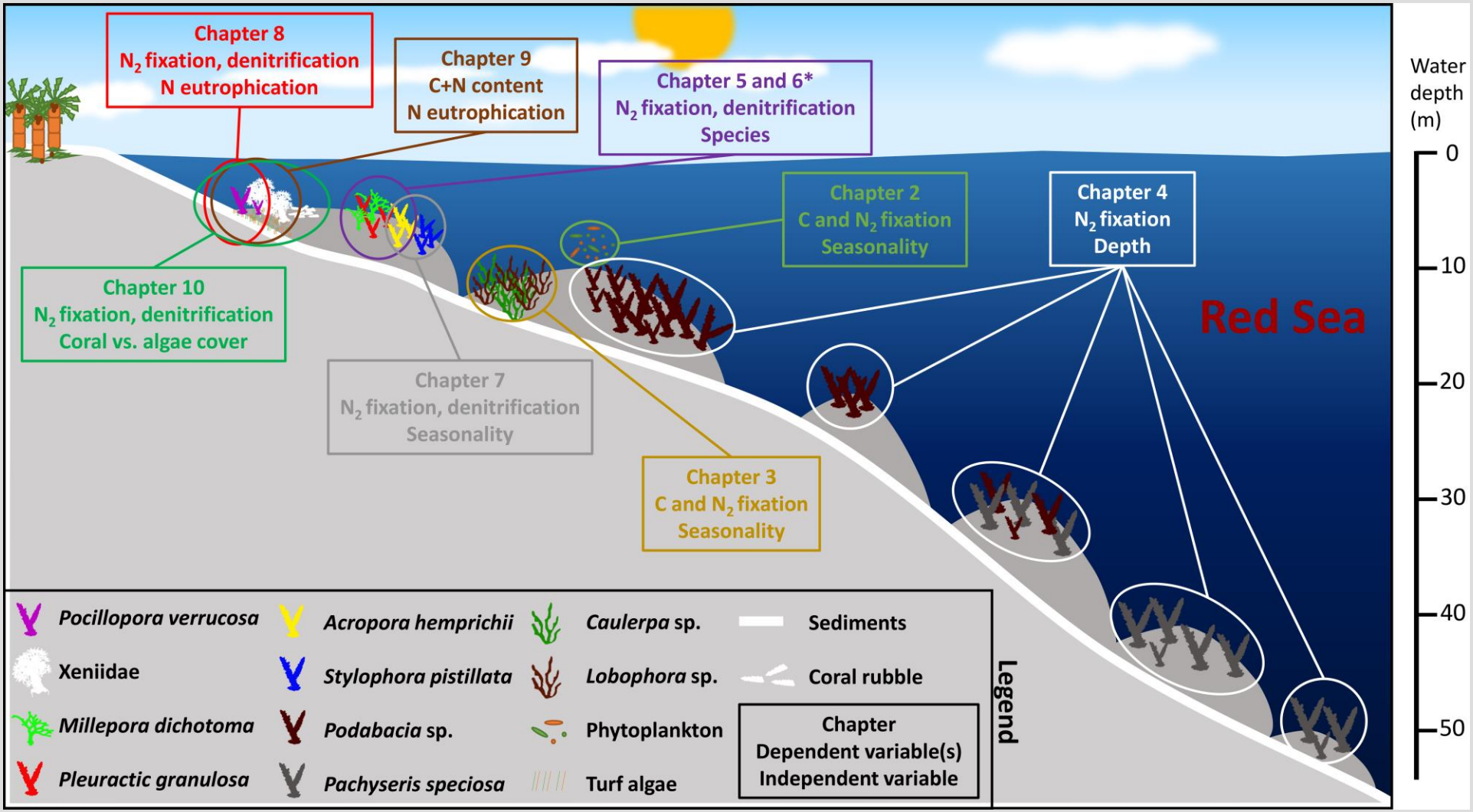

Figure 1.1 | Overview of chapters and the respective dependent and independent variable(s) described in this thesis. All experiments were performed at the central and northern Red Sea. *Chapter 6 is a supplement to Chapter 5.

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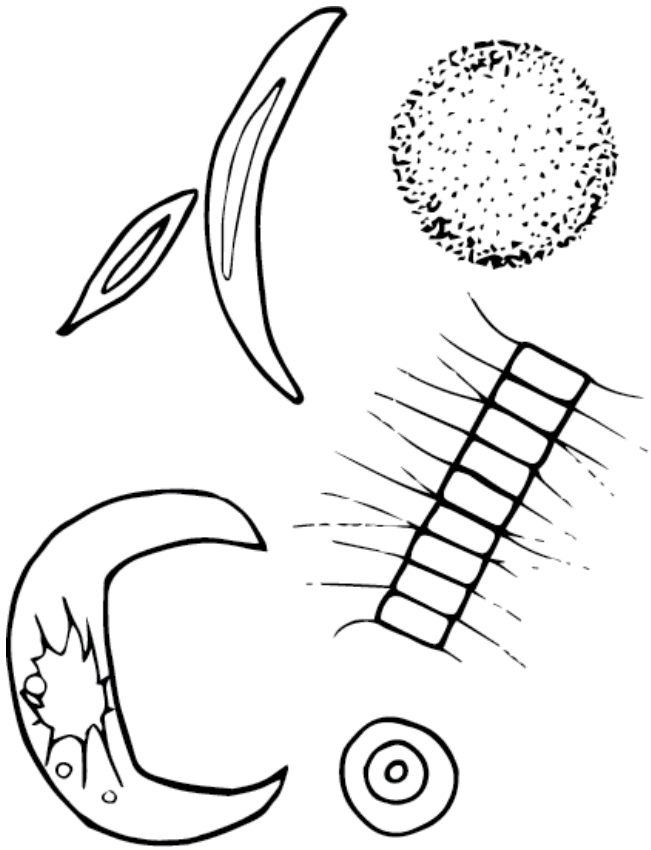
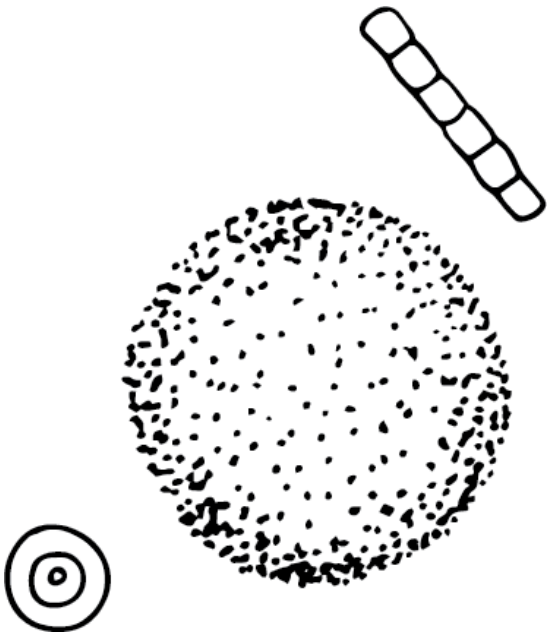


Chapter 2



Chapter 2 | Effects of water column mixing and stratification on planktonic primary production and dinitrogen fixation on a northern Red Sea coral reef

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2.1 | Abstract

The northern Red Sea experiences strong annual differences in environmental conditions due to its relatively high−latitude location for coral reefs. This allows the study of regulatory effects by key environmental parameters (i.e., temperature, inorganic nutrient, and organic matter concentrations) on reef primary production and dinitrogen (N_2) fixation, but related knowledge is scarce. Therefore, this study measured environmental parameters, primary production and N_2 fixation of phytoplankton groups in the water overlying a coral reef in the Gulf of Aqaba. To this end, we used a comparative approach between mixed and stratified water column scenarios in a full year of seasonal observations. Findings revealed that inorganic nutrient concentrations were significantly higher in the mixed compared to the stratified period. While gross photosynthesis and N_2 fixation rates remained similar, net photosynthesis decreased from mixed to stratified period. Net heterotrophic activity of the planktonic community increased significantly during the stratified compared to the mixed period. While inorganic nitrogen (N) availability was correlated with net photosynthesis over the year, N_2 fixation only correlated with N availability during the mixed period. This emphasizes the complexity of planktonic trophodynamics in northern Red Sea coral reefs. Comparing mixed and stratified planktonic N_2 fixation rates with those of benthic organisms and substrates revealed a close seasonal activity similarity between free−living pelagic and benthic diazotrophs. During the mixed period, N_2 fixation potentially contributed up to 3 % of planktonic primary production N demand. This contribution increased by ca. one order of magnitude to 21 % during the stratified period. Planktonic N_2 fixation is likely a significant N source for phytoplankton to maintain high photosynthesis under oligotrophic conditions in coral reefs, especially during stratified conditions.

Keywords: plankton | primary production | dinitrogen fixation | Gulf of Aqaba | diazotrophs

A modified version of this chapter has been published in *Frontiers in Microbiology* 9:2351.
https://doi.org/10.3389/fmicb.2018.02351

2.2 | Introduction

Coral reefs thrive under oligotrophic conditions, particularly due to autochthonous generation of organic carbon (C) and nitrogen (N) through photosynthetic primary production, prokaryotic dinitrogen (N_2) fixation and efficient internal recycling of those materials within the ecosystem[1]. Internal recycling can occur through strong benthic–pelagic coupling of dissolved and particulate organic matter (POM) and nutrients[2–4]. Benthic–pelagic coupling is mediated by benthic organisms such as corals, algae, and sponges[5–7], but also by reef sediment and framework microbiota[8,9]. Furthermore, reefs receive allochthonous energy and nutrients in the form of inorganic nutrients, plankton, and particulate/dissolved matters from offshore and/or riverine inflow. Since reefs are often N limited, diazotrophs (i.e., prokaryotes capable of N_2 fixation) can facilitate primary production, particularly under oligotrophic conditions such as those found in the Gulf of Aqaba[10–12].

The Gulf of Aqaba forms one of the northern tips of the Red Sea. Its desert coasts are bordered by fringing coral reefs that experience relatively strong variation in light availability and water temperature for warm–water coral reefs due to its relative high latitude location. The annual fluctuation in sea surface temperature (21–29 °C) combined with relatively warm deeper water layers (year round ~21 °C for water depth > 200 m) result in an annual cycle of deep water mixing from December until May and stratification down to 200 m water depth from June until ~November[13] with slow destratification from October and onwards[14–16]. Inorganic nutrients are brought up to surface water layers during deep water mixing but are trapped in deeper waters during stratification, creating extreme oligotrophic conditions in coral reef surrounding surface waters[8,17,18]. These local physico–chemical conditions offer the rare opportunity to study the effects of variation in key environmental factors on important processes such as primary production and diazotrophy within coral reefs.

Planktonic primary production in the Gulf of Aqaba and northern Red Sea is dominated by photoautotrophic nano– and picoplankton. The plankton fraction < 20 mm performs on average 81 % of planktonic photosynthesis in the upper water layer (discrete depth)[19] ranging from 0.02 to 3.38 µmol C L^{-1} d^{-1}, assuming a 12 h period of daylight[19,20]. The phytoplankton community in the Gulf of Aqaba is characterized by a strong shift in its composition between annual periods, i.e., mixed and stratified. During the mixed period, eukaryotic algae account for up to 95 % of phytoplankton biomass, while during stratification > 60 % of the biomass consists of prokaryotes, in particular *Prochlorococcus* sp. which may comprise up to 50 % of the biomass[21]. The main groups of planktonic diazotrophs in the Gulf of Aqaba are Cyanobacteria and Proteobacteria[22,23], and they are responsible for water column N_2 fixation rates ranging from 0.01 to 1.9 nmol N L^{-1} d^{-1}. Planktonic photosynthesis rates in reef–surrounding waters can be one to two orders of magnitude higher than in oceanic waters offshore[24,25]. Moreover, primary production and N_2 fixation can interact synergistically within or between planktonic organisms; photosynthesis (and the subsequent organic C released to the surrounding water) may fuel the energy–demanding process of N_2 fixation, which in turn can support primary production by supplying the bioavailable N required to synthesize proteins for photosynthesis[26].

Seasonality in the Gulf of Aqaba affects benthic primary production and N_2 fixation in a range of common organisms and substrates including corals[27,28], algae[29,30], and sediments[31]. Some of these studies found positive relationships between the two processes, as well as with light intensity, temperature and a negative relationship with N availability[27,29,30]. While studies exist for open water planktonic primary production and/or N_2 fixation in the Gulf of Aqaba[22,23,32], to our knowledge, there are no studies relating

these two processes of planktonic communities directly overlying a coral reef, where strong benthic pelagic coupling is evident. Thus, to increase our understanding of the factors regulating planktonic primary production and N_2 fixation, the objectives of this study were (*i*) to measure environmental parameters in a Gulf of Aqaba fringing coral reef over the two distinct periods (i.e., mixed and stratified), (*ii*) to quantify primary production and N_2 fixation of the planktonic community, in water directly overlying a coral reef, over the two periods, (*iii*) to investigate functional relationships between primary production, N_2 fixation and environmental parameters, and (*iv*) to compare the pattern of primary production and N_2 fixation with benthic organisms and substrates investigated in parallel.

2.3 | Materials and Methods

Study site and environmental monitoring

The fieldwork for this study was conducted at the Marine Science Station (MSS) of The University of Jordan, located 10 km south of Aqaba, Jordan. The MSS is situated adjacent to a marine protected area encompassing a crescent shaped fringing coral reef with a length of ca. 1 km. All water column sampling was performed at 10 m water depth in the fore reef section (29° 27′ 31″ N, 34° 58′ 26″ E). Two extensive fieldwork campaigns were performed in 2013: one during the mixed period (January–April; 12 weeks), and one during the stratified period (September–November; 13 weeks). The studied reef consists of a shallow reef flat (< 1 m water depth) surrounded by a carbonate sediment belt at ca. 5 m water depth and a coral dominated middle−fore reef facing the open sea (see Cardini et al.[33] for a visual description of the site).

Light intensity, water temperature, chlorophyll *a* (Chl *a*), dissolved organic carbon (DOC), particulate organic carbon (POC), particulate nitrogen (PN), and inorganic nutrients, i.e., NH_4^+, PO_4^{3-}, NO_2^- and NO_3^-, were monitored during each period (at 10 m water depth). Light intensity measurements recorded by data loggers in lux units were converted to photosynthetically active radiation (PAR) by a conversion factor calculated from a simultaneous minute−by−minute measurement of lux and PAR (08:00–14:00 on 1 day, n = 353) using a HOBO pendant logger and a LI−COR LI192SA underwater quantum sensor: lux = PAR x 52.0, r^2 = 0.83. This value is comparable to the conversion factor given by Valiela[34]: 51.2. PAR values measured per minute were summed for each weekly water−sampling day: values in µmol quanta m^{-2} s^{-1} were recalculated to mol quanta m^{-2} d^{-1}. Water temperature, measured per minute on the water−sampling day, was averaged over the 24 h period. Water samples were collected from 10 m water depth on a weekly basis (during both campaigns) in clean high density poly−ethylene (HDPE) containers (volume: 5 L) using SCUBA. All samples were collected between 08:00 and 10:00 within a 10 min timeframe. Sampling was performed 1 m above the seafloor without disturbing the benthos. Chl *a* and NH_4^+ were measured fluorometrically while remaining parameters (i.e., POC, PN, PO_4^{3-}, NO_2^-, and NO_3^-) were measured photometrically as detailed by Bednarz et al.[31]. In addition, DOC was measured according to Cardini et al.[28] using a Shimadzu TOC−VCPH total organic carbon analyser. For temperature, PAR, nutrient (DIN and PO_4^{3-}) and chl *a* data along the entire depth gradient, see Bednarz et al.[35].

Quantification of primary production

All incubations for primary production and N_2 fixation rates were performed in a flow through mesocosm (~4000 L h^{-1} exchange rate) with water from the reef at 10 m sampling depth to ensure *in situ* conditions (temperature). Light intensity was adjusted to 10 m depth values by using black netting.

Two water subsamples were taken per HDPE container, one for net photosynthesis (P_n) and one for dark

respiration (*R*) measurement. Weekly incubations were performed in individual 1 L closed cell respirometric glass chambers (*n* = 6) under constant stirring (600 rpm). Chambers for *R* measurements were placed in bags made of dense opaque plastic (volume: 10 L) for incubation in the dark. P_n incubations were performed from 10:00 until sunset (ca. 17:00–18:00; depending on the period), while *R* incubations ran for 24 h to get measurable rates and to account for diurnal fluctuations. P_n and *R*–values were obtained by using a conductivity– and temperature–corrected O_2 optode sensor (MultiLine® IDS 3430, WTW, accuracy: ± 0.5 % of measured value) and calculated by subtracting O_2 start from end concentrations. Subsequently, values obtained for P_n and *R* were corrected for incubation duration and chamber volume, and recalculated to molar equivalents resulting in hourly O_2 measurements in µmol O_2 L^{-1} h^{-1}. *R* is presented here as a positive value. Estimates of gross photosynthesis (P_g) were calculated as $P_g = P_n + R$. As *R* is averaged over 24 h, it represents a value assuming a constant hourly rate. Since *R* is susceptible to daily fluctuations, with possible lower *R* during darkness, P_g–values are possibly underestimated. However, *R*–values measured here also includes the heterotrophic component of the community adding to P_g.

To assess the contribution of planktonic C to the water column and C budget of the ecosystem, P_n and *R* rates were recalculated to metabolic C production per day by the following equations, assuming photosynthetic and respiratory quotients of 1.4 and 1.1, respectively[36]. Gross primary production (GPP) = (P_n + *R*) × h of daylight (using the average hourly rate of *R*), calculates the daily fixation of C by autotrophs; community respiration (CR) = *R* × 24 h, calculates daily respiration of the entire community, i.e., auto– and heterotrophs; net community production (NCP) = GPP–CR, calculates whether the system is net autotrophic or heterotrophic over the 24 h. Values presented as µmol C L^{-1} d^{-1}. Finally, daily contribution of planktonic C to total organic C (TOC = DOC + POC) was calculated by dividing GPP, NCP, or CR with TOC, and multiplying by 100 to obtain percentages.

Quantification of N_2 fixation

Planktonic N_2 fixation rates were measured during the mixed and stratified periods using a modified acetylene (C_2H_2) reduction technique[37,38]. Weekly incubations were performed in 1 L chambers (*n* = 8) containing 800 mL seawater and a 200 mL headspace both being 10 % C_2H_2–enriched under constant stirring (600 rpm). Control incubations were performed without C_2H_2 addition to measure biological ethylene (C_2H_4) production, as well as with sterile filtered seawater (0.2 µm) to measure inorganic C_2H_4 production from C_2H_2. The incubations lasted for 24 h, and 1 mL gas samples were extracted from a port in the lid at 0, 4, and 24 h with a gastight syringe and analyzed for C_2H_4 concentration using a customized reducing compound photometer (Peak Laboratories, Mountain View, CA, United States, detection limit = 100 ppb). C_2H_4 measurements were recalculated to nmol C_2H_4 in the whole chamber water volume. It was determined in pilot experiments that the C_2H_2 concentration equilibrated between headspace and incubation water in the first 4 h, the C_2H_4 production over 4 to 24 h were therefore used for calculations. Changes in C_2H_4 concentration over time were corrected for incubation duration and volume of water in the chamber, resulting in measurements of nmol C_2H_4 L^{-1} h^{-1}. Due to the ongoing debate concerning conversion factors, C_2H_4 evolution rates measured here are used as a proxy for estimated N_2 fixation rates and are thus not converted to actual nitrogen fixed[38]. Only for estimating the contribution of N_2 fixation to the N requirement of primary production, C_2H_4 rates were converted to N m^{-3} d^{-1} using the conservative theoretical C_2H_4:N_2 conversion ratio of 4:1[39].

Statistical analyses

The values of replicate measurements for environmental parameters, primary production and N_2 fixation for each week were averaged prior to statistical analyses. All statistics were performed in Sigmaplot 12.0 for Windows (Systat software). Environmental parameters, primary production and N_2 fixation during the two periods were tested for normality with the Shapiro–Wilk test. Comparisons between the two periods were performed with independent samples *T*–tests if data were normally distributed and with Mann–Whitney *U*–tests if data lacked normality.

In addition, relationships between N_2 fixation rates, P_n, P_g, and *R* rates per period and across both periods with environmental water parameters were determined via linear regression. Differences were deemed significant at $p < 0.05$. All values are given as mean ± SE.

2.4 | Results

Environmental variables

Mean weekly measurements of environmental parameters were variable over time (Figure 2.1). Daily PAR increased from January to April (i.e., mixed period) from 3.45 ± 0.26 to 5.76 ± 0.11 mol quanta m^{-2} d^{-1}, and decreased again from September to November (i.e., stratified period) from 6.72 ± 0.37 to 4.25 ± 0.38 mol quanta m^{-2} d^{-1} (Figure 2.1A). However, between periods no significant differences were found for PAR (Table 2.1). Temperature was stable throughout the mixed period (22.1–22.9 °C), but increased to a maximum of 27.5 °C in early September followed by a decrease to 24.7 °C at the end of November. Inorganic nutrient and Chl *a* concentrations were all significantly lower during the stratified than during the mixed period (Figure 2.1B and Table 2.1, all $p < 0.002$), while DOC was significantly higher during the stratified period than during the mixed period (Figure 2.1C and Table 2.1). POC concentrations showed no significant differences between periods, while PN was significantly lower in the stratified period (Figure 2.1C), causing a significantly higher POC:PN ratio (Table 2.1). The DIN:PO_4^{3-} ratio was not significantly different between periods (Table 2.1).

Table 2.1 | Environmental water parameters in the mixed and stratified periods.

Parameters	Mixed	Stratified	*p*
PAR (mol m^{-2} d^{-1})	4.71 ± 0.38	5.39 ± 0.44	0.264
Temperature (°C)*	22.34 ± 0.07	25.84 ± 0.28	**<0.001**
Chl *a* (µg L^{-1})	0.20 ± 0.01	0.14 ± 0.01	**0.002**
DOC (µmol L^{-1})	75.51 ± 2.10	87.36 ± 1.17	**<0.001**
POC (µmol L^{-1})	7.90 ± 0.86	7.74 ± 0.50	0.865
TOC (µmol L^{-1})*	83.51 ± 2.32	95.10 ± 1.22	**<0.001**
PN (µmol L^{-1})*	1.08 ± 0.08	0.88 ± 0.05	**0.024**
NH_4^+ (µmol L^{-1})*	0.52 ± 0.06	0.25 ± 0.03	**<0.001**
PO_4^{3-} (µmol L^{-1})	0.11 ± 0.00	0.04 ± 0.01	**<0.001**
NO_x (µmol L^{-1})	0.63 ± 0.08	0.19 ± 0.04	**<0.001**
POC:PN	7.16 ± 0.39	8.77 ± 0.34	**0.005**
DIN:PO_4^{3-}*	10.42 ± 0.70	16.59 ± 3.14	0.717

Differences were tested with independent sample t–tests, except for parameters with *, these were tested with Mann–Whitney U–tests due to lack of normality. Chl *a* = chlorophyll a, DOC = dissolved organic carbon, POC = particulate organic carbon, TOC = total organic carbon (POC + DOC), PN = particulate nitrogen, $NO_x = NO_2^- + NO_3^-$, DIN = $NH_4^+ + NO_x$. Values are given as mean ± SE. Bold p–values indicate significant values.

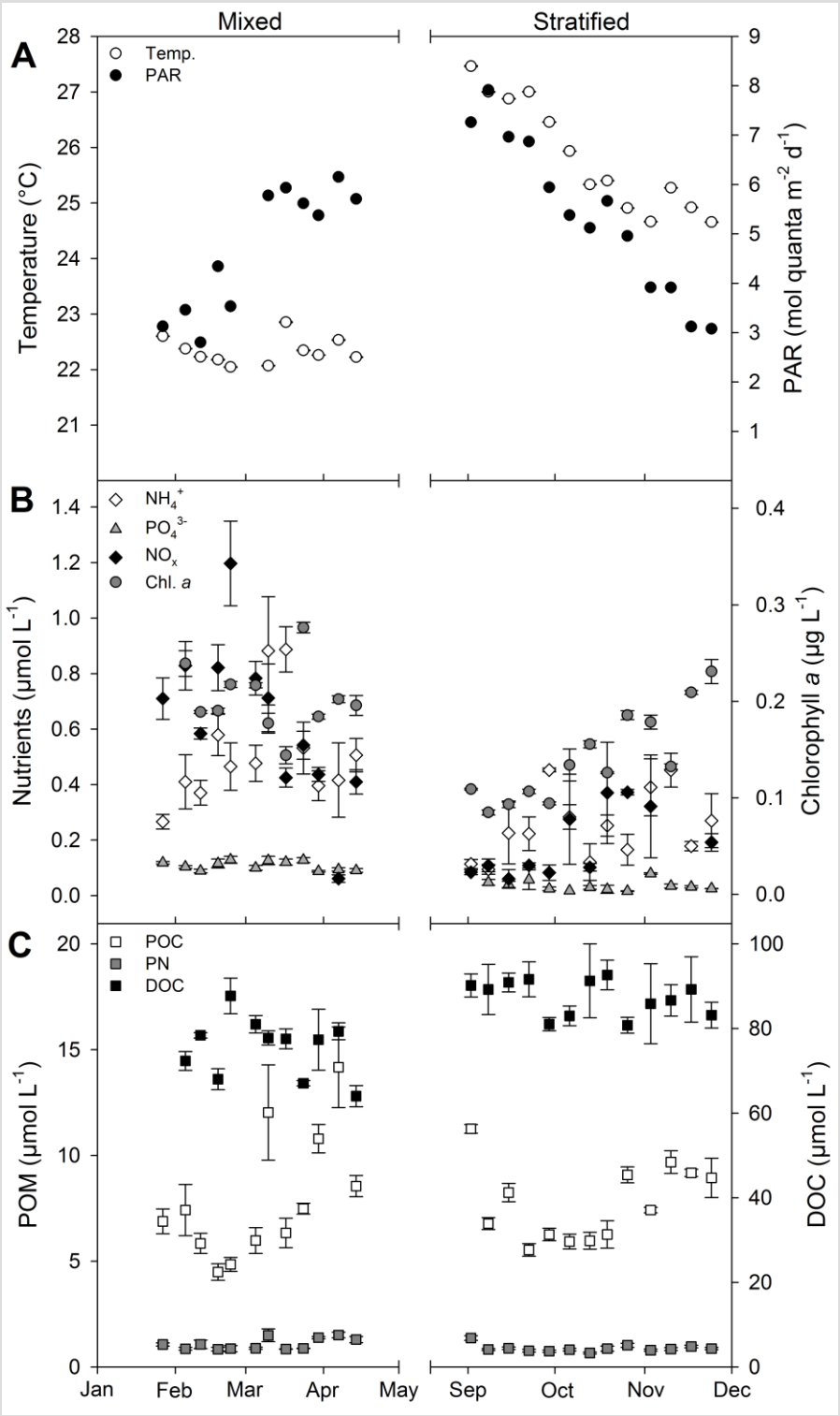

Figure 2.1 | Mean weekly environmental water parameters measured at 10 m water depth (1 m over coral reef benthos) during the mixed and stratified periods. **(A)** Temperature and PAR. **(B)** Nutrients and chlorophyll *a*. **(C)** POM and DOC. Values given as mean ± SE. Temp = water temperature, PAR = photosynthetically active radiation measured at 10 m water depth, NO_x = NO_2^- + NO_3^-, POC = particulate organic carbon, PN = particulate nitrogen, POM = particulate organic matter (POC and PN), DOC = dissolved organic carbon.

Primary production

Mean water column P_n was always negative (between –0.54 and –0.09 µmol O_2 L^{-1} h^{-1}), except on 18 February and 3 March (0.00 and 0.02 µmol O_2 L^{-1} h^{-1}, respectively), and significantly lower during the stratified period than the mixed period (Figure 2.2 and Table 2.2). *R* ranged from 0.15 to 0.46 µmol O_2 L^{-1} h^{-1} and was not significantly different between periods. Estimates of P_g were higher for the mixed period compared to the stratified period with averages of 0.09 and 0.02 µmol O_2 L^{-1} h^{-1}, respectively. However, these estimates were not significantly different (Table 2.2). GPP and CR was relatively stable and not significantly different between periods (Table 2.2 and Figure 2.3). Mean water column TOC was 83.51 and 95.10 µmol L^{-1} d^{-1} for the mixed and stratified periods, respectively (Table 2.1). The contribution of net daily planktonic C (NCP) to TOC was comparable during the mixed and stratified periods, 7.25 ± 0.95, and 7.73 ± 0.49 % d^{-1}, respectively (Figure 2.3).

N_2 fixation activity

Weekly mean N_2 fixation ranged from 0.18 to 0.71 and 0.07 to 1.28 nmol C_2H_4 L^{-1} h^{-1} in the mixed and stratified periods, respectively (Figure 2.2). Although mean N_2 fixation on 8 and 22 September, during stratification, were substantially higher than during the mixed period, overall, there was no significant difference (Table 2.2).

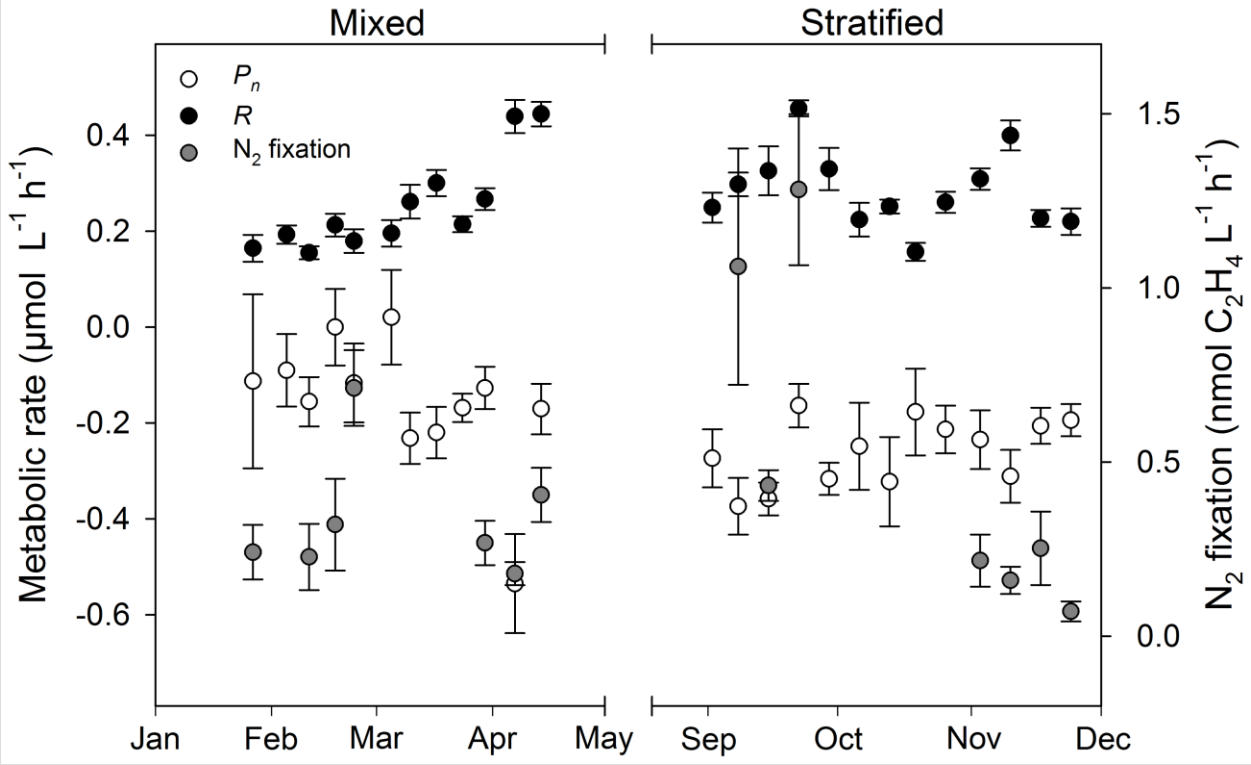

Figure 2.2 | Planktonic primary production and N_2 fixation rates measured weekly at 10 m water depth (1 m above the benthos) during the mixed and stratified periods. P_n = net photosynthesis, *R* = dark respiration. Values given as mean ± SE.

Table 2.2 | Primary production (P_n, R and P_g, GPP, CR and NCP) and N_2 fixation in the mixed and stratified periods.

Parameters	Mixed	Stratified	*p*
P_n (µmol O_2 L^{-1} h^{-1})*	−0.16 ± 0.04	−0.26 ± 0.02	**0.004**
R (µmol O_2 L^{-1} h^{-1})*	0.25 ± 0.03	0.29 ± 0.02	0.165
P_g (µmol O_2 L^{-1} h^{-1})	0.09 ± 0.03	0.02 ± 0.03	0.094
GPP (µmol C L^{-1} d^{-1})	0.78 ± 0.26	0.19 ± 0.23	0.094
CR (µmol C L^{-1} d^{-1})*	6.66 ± 0.75	7.52 ± 0.59	0.165
NCP (µmol C L^{-1} d^{-1})*	−5.87 ± 0.78	−7.33 ± 0.46	**0.036**
N_2 fixation (nmol C_2H_4 L^{-1} h^{-1})	0.34 ± 0.07	0.50 ± 0.18	0.423

Differences were tested with independent sample t−tests, except for parameters with *, these were tested with Mann−Whitney U−tests due to lack of normality. P_n = net photosynthesis, R = dark respiration, P_g = gross photosynthesis = P_n + R, GPP = gross primary production, CR = community respiration, NCP = net community production over the day = GPP – CR. Values are given as mean ± SE. Bold p−values indicate $p < 0.05$.

Relationship analyses

Positive relationships were revealed for P_n and availability of N (NO_x and/or DIN) during the mixed and stratified period and when both periods were combined (Table 2.3). Additionally, negative relationships were revealed for P_n with temperature and PAR. R revealed a negative relationship with NO_x during the mixed period and when both periods are combined (Table 2.3). No relationships were found for P_g with any environmental parameter (Table 2.3). Positive relationships for N_2 fixation and N availability were found for the mixed period while for the stratified period positive relationships were established with both temperature and PAR. A positive relationship was established for PAR when periods were combined (Table 2.3). Additionally, no relationships were found between any of the primary production parameters and N_2 fixation (not shown in Table 2.3).

Table 2.3 | Linear regression analysis (r^2 values) examining the relationships between the physiological parameters.

	Temp	PAR	NO_x	DIN
P_n				
Mixed	0.178	0.307	**0.510***	**0.377***
Stratified	0.177	0.186	**0.377***	0.324
Combined	*0.208**	*0.255**	**0.544*****	**0.395****
R				
Mixed	0.038		*0.550***	0.257
Stratified	0.133		0.252	0.117
Combined	0.060		*0.327***	0.168
P_g				
Mixed	0.063	0.001	0.073	0.064
Stratified	0.000	0.013	0.003	0.022
Combined	0.082	0.014	0.147	0.148
N_2 fixation				
Mixed	0.507	0.038	**0.573***	**0.640***
Stratified	**0.716***	**0.700***	0.302	0.409
Combined	0.248	**0.406***	0.016	0.057

P_n = net photosynthesis, R = dark respiration, P_g = gross photosynthesis, N_2 fixation = dinitrogen fixation, Temp = temperature, PAR = photosynthetically active radiation, DIN = dissolved inorganic nitrogen, NO_x = NO_2^- + NO_3^-, DIN = NH_4^+ + NO_x. Bold values indicate significant positive relationships, and italicized values indicate significant negative relationships. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.5 | Discussion

Studies relating planktonic N_2 fixation and primary production in the highly seasonal Gulf of Aqaba in the northern Red Sea are, to our knowledge, only related to open water settings[23]. Rahav et al.[23] highlighted the need for both spatial and temporal sampling of planktonic communities in the Gulf of Aqaba to disentangle the complex dynamics of these communities. In this study, water samples taken 1 m above the reef benthos were used to measure planktonic primary production and N_2 fixation rates on a weekly basis during both the mixed and the stratified period. Findings revealed significantly higher primary production (P_n) during the mixed period (Table 2.2), while planktonic N_2 fixation rates remained stable between both periods (Table 2.2). Similar to open water, higher net heterotrophic activity was measured during the stratified compared to the mixed period, but the open water column remained net autotrophic. Moreover, Rahav et al.[23] found a potential coupling between pelagic primary production and N_2 fixation only during the mixed period, while linear regression analyses supported no such relationship here. However, results obtained here do suggest that N_2 fixation, as estimated by C_2H_4 evolution, has the potential to maintain stable pelagic GPP throughout the year by contributing ~6 times more bioavailable N for GPP during the N depleted stratified period compared to the mixed period.

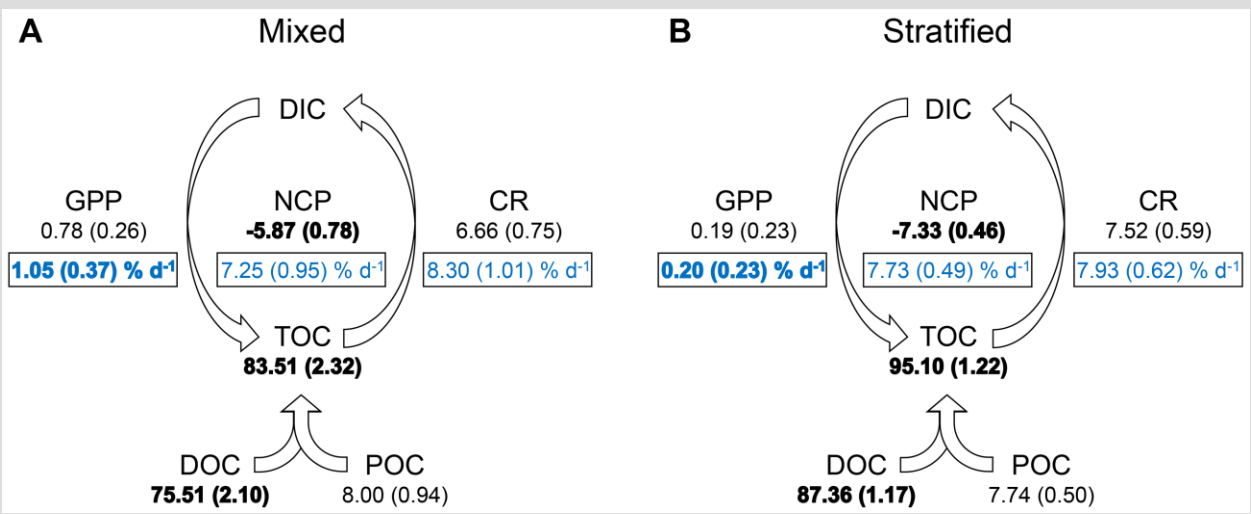

Figure 2.3 | Mean daily planktonic carbon balance in mixed (A) and stratified (B) periods. GPP = gross primary production, CR = community respiration, NCP = net community production (GPP – CR). Values given as mean (SE). GPP, CR, NCP in µmol C L^{-1} d^{-1}. DOC = dissolved organic carbon, TOC = total organic carbon (POC (particulate organic carbon) + DOC), TOC, DOC and POC in µmol C L^{-1}, DIC = dissolved inorganic carbon. Boxed light blue text constitutes the percentage of planktonic C contributed daily to TOC (mean (SE)). Bold indicates a significant difference between periods ($p < 0.05$).

Environmental parameters in mixed and stratified period

Photosynthetically active radiation, water temperature, and inorganic nutrient concentrations during the mixed and stratified periods were comparable to previous research at the study site[6,8]. The average mixed period DIN:PO_4^{3-} ratio (10.42) was lower than the Redfield ratio for N:P (16:1)[40], suggesting N as the limiting nutrient. The average N:P ratio in the stratified period was similar to the Redfield N:P ratio, indicating that inorganic N and P availability in that period was on average balanced. However, ratios

between 31 and 33 found in three non−consecutive weeks in October and November suggest that there were times when P may have been limiting. Previous studies have also found that both N and P are limiting factors for primary production in the northern Red Sea[41–43]. Chl *a* decreased significantly from the mixed to the stratified period, coinciding with declining inorganic nutrients and increasing water temperature and PAR. A strong negative relationship with PAR was found especially during the stratified period (r^2 = 0.776, $p < 0.001$). Indeed, higher PAR often causes a reduction in Chl *a* in phytoplankton[44], but this response is specific per species[45]. Furthermore, Gittings et al.[46] found a strong, near perfect, negative correlation between Chl *a* and sea surface temperature in the northern Red Sea. This is also confirmed by our data as a strong negative relationship was found between Chl *a* and temperature when both periods were combined (r^2 = 0.597, $p < 0.001$). Average POC:PN ratios in the mixed and stratified period were higher than Redfield proportions (106:16 = 6.625), 7.16 and 8.77, respectively, suggesting that the POM in the water column was impoverished in N throughout the year, particularly so during the stratified period when PN concentrations were significantly reduced. The dominant source of POM in coral reef−surrounding waters can be mucus released by hard corals[47,48]. Mucus POC and PN release by the dominant hard corals in the studied reef is constant over the year[6]. However, the average POC:PN ratio of coral mucus (12 ± 1)[6], is far higher than the periodic ratios found for our water samples, indicating that a large fraction of water column PN originated from another source subject to differences in environmental conditions. This is confirmed by Hadas et al.[49], who found that the majority of water column PN in a Gulf of Aqaba reef consisted of or was produced by pelagic prokaryotes. In addition, DOC measured in our water samples was significantly higher in the stratified period (87.36 µmol L^{-1}) compared to the mixed period (75.51 µmol L^{-1}). DOC may be more abundantly released by benthic coral reef algae than by co−occurring hard corals[50,51]. DOC release by turf algae and the algal genus *Peyssonnelia* in the studied reef is indeed higher during the stratified than during the mixed period[5]. A positive relation between DOC release and temperature is common in marine macrophytes[52]. Thus, increased benthic release could explain the increased water column DOC concentration found during the stratified period.

Table 2.4 | Comparison of daily gross primary production (GPP) rates from this study and literature references.

Location	GPP (µmol C L^{-1} d^{-1})	Reference
Gulf of Aqaba	0.00 – 2.54†	This study
Gulf of Aqaba	0.05 – 3.38*	Levanon−Spanier et al.[20]
Gulf of Aqaba (open surface water)	0.02 – 0.26	Rahav et al.[23]
Northern Red Sea	0.02 – 3.00*	Qurban et al.[19]
Great Barrier Reef, Australia	0.80 – 3.33	Sorokin[53]
Vietnam	0.15 – 3.00	Tac−An et al.[54]
Hawaii	2.01 ± 0.17† (mean ± SE)	Johnson and Wiegner[55]
New Caledonia	0.47 ± 0.05 (mean ± SE)	Torréton et al.[56]

† recalculated from O_2 values using a photosynthetic quotient of 1.4[36]. * extrapolated to per day assuming 12 h of daylight.

Planktonic primary production and N_2 fixation

The planktonic GPP rates measured in the mixed and stratified periods fall within the range previously recorded for the Gulf of Aqaba[20] and rates measured for other reef−surrounding waters worldwide (Table 2.4). However, compared to open surface waters of the Gulf of Aqaba, GPP found here was three− to fourfold higher during the mixed period and four− to ninefold higher during the stratified period[23]. NCP was always negative, indicating that the planktonic community as a whole acted net heterotrophically,

particularly during the stratified period[33]. Planktonic communities in waters surrounding coral reefs are often net heterotrophic and are likely fueled by a steady supply of organic matter released from the reef benthos[50,57]. Even though PAR and temperature were both negatively related with P_n, the strong positive relationship of P_n with both DIN and/or NO_x within each period and combined indicated that inorganic N, rather than PAR or temperature, was the strongest environmental parameter that limited P_n in these oligotrophic waters.

Dinitrogen fixation rates measured in this study were within the range found for planktonic communities from different locations worldwide (Table 2.5). Our maximum rates appear high compared to most literature values, but this is mainly due to two high values measured in September (1.06 and 1.28 nmol C_2H_4 L^{-1} h^{-1}). The other weekly mean values in the present study average 0.29 ± 0.05 nmol C_2H_4 L^{-1} h^{-1}, which falls within the range of most literature values (0.004–0.46 nmol C_2H_4 L^{-1} h^{-1}). These values, like the values in this study, were found in the upper water column up to 10 m depth, while higher values (~2.75 and 0.87 nmol C_2H_4 L^{-1} h^{-1}) were measured at depths > 25 m[38,58]. Measured values from the tropical Atlantic Ocean (up to 1.04 C_2H_4 L^{-1} h^{-1} [59], assuming the conservative theoretical C_2H_4:N_2 conversion ratio of 4:1[39]), indicate that high rates, such as those found in September, are possible under comparably oligotrophic conditions. Moreover, the high September N_2 fixation rates coincided with the lowest DIN concentrations of all sampling occasions (0.20–0.29 µmol L^{-1}). N_2 fixation is energy−costly and many diazotrophs can increase their N_2 fixation in times of inorganic/organic N scarcity[60]. However, this potential negative relationship could not be evidenced with linear regression. On the contrary, a positive relationship was found during the mixed period while no relationship was found during the stratified period. A large part of the DIN measured during this study consisted of NO_x. NO_3^- in particular has a lower inhibitory effect on nitrogenase (the enzyme responsible for N_2 fixation), compared to NH_4^+, depending on, e.g., light or PO_4^{3-} availability[61–63]. Thus, certain abiotic factors may mitigate the nitrogenase inhibiting effects of NO_3^-. Alternatively, ambient DIN concentrations may not be high enough to inhibit nitrogenase[60]. During the stratified period, when temperature was highest and the highest PAR values were measured, N_2 fixation rates coincided with these parameters. However, when combined, PAR was the environmental parameter that best explained N_2 fixation rates found throughout the year suggesting a prominent role for photoautotrophic diazotrophs. This coincides with relationships found for other organisms and substrates[30,31].

Table 2.5 | Comparison of hourly dinitrogen (N_2) fixation rates from this study and literature references (given as ranges) measured by acetylene reduction assay.

Location	N_2 fixation (nmol C_2H_4 L^{-1} h^{-1})	Reference
Gulf of Aqaba	0.07 – 1.28	This study
North Pacific Ocean	~0.06 – 0.08	Mague[64]
Central North Pacific Ocean	0.004 – 0.098	Mague et al.[65]
Central Arabian Sea	~0.15	Capone et al.[66]
Western North Pacific Ocean	~0.01 – 0.46	Kitajima et al.[67]
Northeast Atlantic Ocean	< 0.03	Benavides et al.[68]
Hawaii	~1.10 – 2.75*	Wilson et al.[38]
Northern South China Sea	0.10 – 0.87	Wu et al.[58]

* assuming a 12:12h light:dark cycle

While GPP remained stable between both periods, the decrease in NCP found in the present study indicates that the planktonic community became more heterotrophic in the stratified period compared to the mixed period. However, this observation is contrasted by linear relationships (Table 2.3), which show that N_2 fixation rates during the stratified period coincided with light availability (indicating autotrophic dominance) rather than DOC availability (not shown in Table 2.3) despite the higher availability of DOC. The benthos of the study site was primarily dominated by primary producers[33,69], many of which release a significant fraction of their photosynthetically fixed carbon as DOC in the surrounding waters[5,6,70]. Since our water samples were taken 1 m above the benthos, DOC would have been available to be consumed by the bacterioplankton, as previously shown in Haas et al.[70], potentially causing an increase in heterotrophic abundance and/or activity. While Chl *a* concentration per planktonic cell could have increased due to increased temperature[71] from the mixed to the stratified period, Chl *a* concentrations (measured per liter) found here decreased with increasing temperature, lending support that less autotrophs were present in the planktonic community during the stratified period. Moreover, Rahav et al.[23] showed a shift toward a more heterotrophic diazotroph community from the mixed to the stratified period while Al−Najjar et al.[21] also showed an increase in *Prochlorococcus* sp. abundance in the picophytoplankton community. Thus, it is likely that the planktonic community during the stratified period contained heterotrophic diazotrophs with lower N_2 fixation rate capacity that was compensated for by higher abundances of *Prochlorococcus* sp. However, a full assessment of the planktonic community, including the identification of the picophytoplankton community, could shed light into this apparent contradiction. Additionally, the potential increase in *Prochlorococcus* sp.[21] may explain why a relationship between P_g and PO_4^{3-} could not be established, despite DIN:PO_4^{3-} ratios in the stratified period regularly exceeding Redfield (with values up to 33). Foster et al.[22] also did not detect P limitation of N_2 fixation and attributed this to the relatively small size of the N_2 fixing microbes in the Gulf of Aqaba, allowing maintenance of N_2 fixation at very low P availability. Small cell size theoretically results in increased nutrient uptake affinity due to allometrically higher surface area to volume ratio, which may have allowed *Prochlorococcus* sp. to maintain photosynthesis under extremely low PO_4^{3-} availability[72]. However, nutrient limitation of planktonic processes is likely more complicated than indicated by the canonical Redfield ratio[73].

Assuming the Redfield C:N ratio (6.625), planktonic N_2 fixation had, on average, the potential to contribute 3.42 % of the N needed for daily GPP during the mixed period. Remarkably, the average potential contribution during stratification was substantially higher as N_2 fixation generated 20.84 % of the potential N demand by GPP. This more than sixfold higher potential contribution indicates that N_2 fixation is a substantial source of N for maintaining stable GPP by the autotrophic community as a whole during extremely oligotrophic conditions in the Gulf of Aqaba. Moreover, the percentage contribution calculated in the present study are similar to those under comparable oligotrophic scenarios in other regions[58,74,75].

Comparison with parallel investigated organisms

Dinitrogen fixation and net photosynthesis of organisms and substrates were previously examined in a seasonal resolution in parallel with the current study[27–31]. For comparisons, raw data of each organism or substrate was averaged after combining winter and spring data to represent the mixed period, and summer and autumn to represent the stratified period.

Comparing the pattern of planktonic N_2 fixation rates between mixed and stratified periods with those of benthic N_2 fixers revealed that, with the exception of *Pocillopora* sp. and both investigated soft corals (i.e., Xeniidae and *Sarcophyton* sp.), symbiotic diazotrophs (those with a eukaryotic host) are significantly affected by seasonal mixing and stratification, while planktonic diazotrophs were not (Table 2.6). The scleractinian coral *Pocillopora* sp. has recently been shown to have an inflexible microbiome in response to environmental stress[76]. This could explain the deviation from the other three scleractinian coral genera as the diazotrophic community could remain relatively stable throughout the year. Furthermore, higher activity of resident diazotrophs is unlikely since abundance and activity of diazotrophs in Red Sea originating *Pocillopora* sp. have been shown to correlate positively[77]. Strikingly, soft corals possessing symbiotic diazotrophs[27], also show no differences between both periods (Table 2.6). Soft corals lack a hard calcium carbonate skeleton and a significant part of a soft corals' weight can consist of water (~80 %)[78]. In this study, planktonic samples were taken 1 m above the reef benthos. Thus, soft corals can potentially take up water holding the same community of planktonic diazotrophs found here by replenishing their water content regularly[79]. Together with its core coral microbiome[80], which might be distinct from its surrounding seawater[81], this could potentially alter their total diazotrophic activity to mimic that of the planktonic community described here. While a similar case could be made for the sponge *Mycale* sp., research has shown that between 72 and 93 % of plankton is grazed and metabolized resulting in 74 % of total daily C intake[82], while in corals, up to 95 % of daily C is translocated from their photosynthetic endosymbiont[83] (belonging to the family Symbiodiniaceae[84]) suggesting a low need for grazing the diazotrophs that are taken up with the surrounding water.

Table 2.6 | Comparison of N_2 fixation and net primary production associated with different coral reef organisms and substrates from the Gulf of Aqaba.

Reef organism/substrate	N_2 fixation activity			Net primary production			Reference
	Mixed	Stratified	*p*	Mixed	Stratified	*p*	
Unknown planktonic diazotrophs	8.07 ± 1.64	11.92 ± 4.34	0.423	−3.81 ± 0.98	−6.27 ± 0.36	**0.004***	This study
Acropora sp.	0.36 ± 0.11	4.65 ± 1.53	**0.002***	14.44 ± 0.78	13.14 ± 1.01	0.323	Cardini et al.[28]
Stylophora sp.	1.84 ± 0.45	5.71 ± 1.63	**0.027***	14.09 ± 1.13	10.77 ± 0.94	**0.0318**	
Pocillopora sp.	2.23 ± 0.44	3.64 ± 0.82	0.250*	11.11 ± 1.32	11.93 ± 1.27	0.655	
Goniastraea sp.	1.78 ± 0.42	10.15 ± 1.93	**<0.001***	14.91 ± 1.11	14.58 ± 1.04	0.825	
Turf algae	53.10 ± 6.12	159.82 ± 29.09	**<0.001***	10.86 ± 0.52	14.27 ± 1.36	0.080*	Rix et al.[29]
Mycale sp.	1.15 ± 0.34	7.77 ± 1.81	**<0.001***	−3.76 ± 0.50	−7.78 ± 1.05	**<0.001***	
Xeniidae	0.17 ± 0.03	0.75 ± 0.20	0.184*	10.16 ± 0.84	8.58 ± 0.49	0.114	Bednarz et al.[27]
Sarcophyton sp.	0.89 ± 0.23	1.87 ± 0.48	0.154*	4.88 ± 0.63	5.31 ± 0.79	0.665*	
Carbonate sand	73.45 ± 15.96	65.52 ± 12.64	0.677*	6.49 ± 1.63	4.23 ± 0.50	0.376*	Bednarz et al.[31]
Silicate sand	37.40 ± 6.01	35.50 ± 4.10	0.795	–	–	–	
Microbial mat	334.66 ± 51.50	241.92 ± 15.65	0.395*	16.94 ± 1.51	15.51 ± 1.30	0.471	
Caulerpa sp.	24.36 ± 10.50	27.26 ± 4.48	**0.031***	23.75 ± 2.38	19.29 ± 1.29	0.116	Tilstra et al.[30]
Lobophora sp.	9.10 ± 3.20	33.81 ± 7.16	**<0.001***	7.73 ± 0.54	6.69 ± 0.45	0.144	

Planktonic diazotroph N_2 fixation and Net primary production in nmol C_2H_4 L^{-1} d^{-1} and µmol O_2 L^{-1} d^{-1}, respectively. All other values for N_2 fixation and Net primary production are given in nmol C_2H_4 cm^{-2} d^{-1} and µmol O_2 cm^{-2} d^{-1}, respectively. Comparison data was obtained by averaging raw values of parallel investigated organism and substrates from winter and spring (mixed period) and those from summer and autumn (stratified period). Differences were tested with independent sample t−tests, except for p−values with *, these were tested with Mann−Whitney U−tests due to lack of normality. Bold *p*−values indicate $p < 0.05$.

Particulate nitrogen of all investigated organisms and substrates remained relatively stable with the exception of *Stylophora* sp. and *Mycale* sp. The latter due to high respiration rates measured during summer and autumn[29]. In general, the pattern of P_n of the planktonic (free living) diazotrophs differed from the benthic (symbiotic) diazotrophs (Table 2.6).

Concluding remarks

Primary production in the water column directly overlying a coral reef in the Gulf of Aqaba appears to be primarily regulated by inorganic N availability, driven by mixing conditions. While inorganic N concentrations declined due to stratification, daily contribution of planktonic GPP to TOC declined significantly while GPP itself remained relatively stable. Compared to open water GPP[23], GPP measured here was between three– and ninefold higher suggesting potential benthic coupling. NCP was significantly more heterotrophic during stratification. However, the daily contribution of NCP to TOC was similar between the two periods due to increased DOC concentrations. The maintenance of biological activity in the water column due to increased DOC availability in times of reduced GPP highlights the importance of the microbial loop in planktonic trophodynamics in these waters[85,86].

In addition to the change in the C budget, there were indications of a potential shift in the N_2 fixation community toward higher heterotrophic activity, similar to findings of Rahav et al.[23]. Causes for this shift could be the decline in inorganic nutrients as well as the increased DOC concentration providing a competitive advantage to heterotrophic diazotrophs[43]. This increase in DOC could be attributable to increased release by benthic turf– and macroalgae[50,51]. Algal–derived organic matter may also promote a more heterotrophic planktonic community than organic matter released by hard corals[70,87]. During stratification, when GPP is strongly nutrient–limited, N_2 fixation shows the potential to contribute a substantial fraction of the N needed, by compensating for low DIN, to maintain stable GPP in the water column.

Dinitrogen fixation was maintained at comparable rates in both periods. Moreover, the results obtained here strongly suggest that N_2 fixation is an important source of N to planktonic primary production. In addition, DOC appears to play an important role in the dynamics of planktonic C and N production/consumption. Further investigation into DOC dynamics through coral reefs is warranted to unravel its effect on energy and nutrient cycles in coral reefs and their surrounding waters. Finally, the findings presented here may be applied to lower latitude coral reefs where the more stable environmental conditions make the disentanglement of driving environmental parameters more complicated.

2.6 | Acknowledgments

We would like to thank S. Basuoni and S. Helber for assistance in the field, as well as C. Staschok, M. Birkicht, and D. Dasbach for field work preparation and sample analyses. This paper is an adapted version previously published in the Ph.D. thesis of van Hoytema[88]. This work was funded through German Research Foundation (DFG) grants Wi 2677/6–1 and Wi 2677/9–1 to CW. VB was funded by a stipend of "Evangelisches Studienwerk Villigst e.V."

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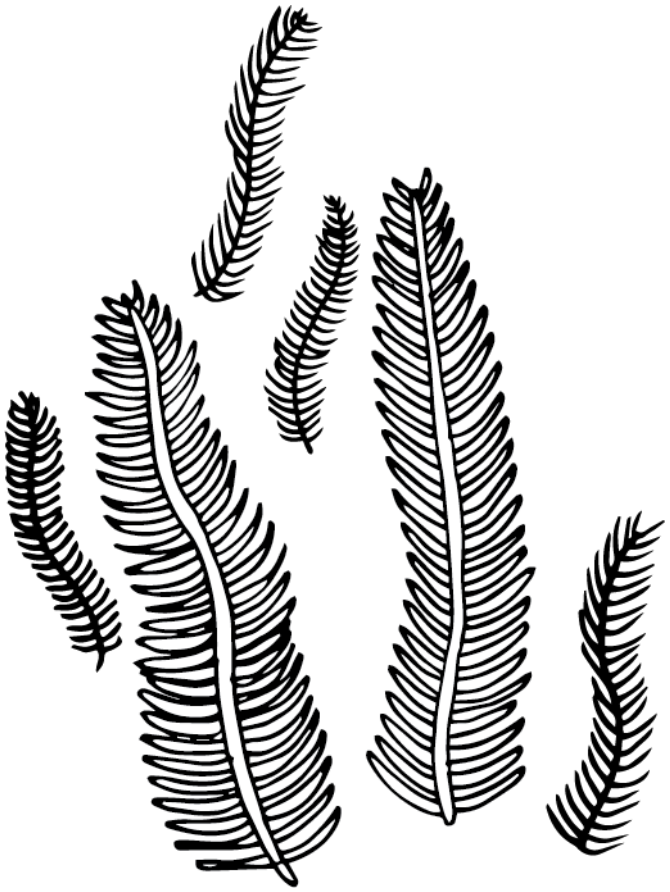

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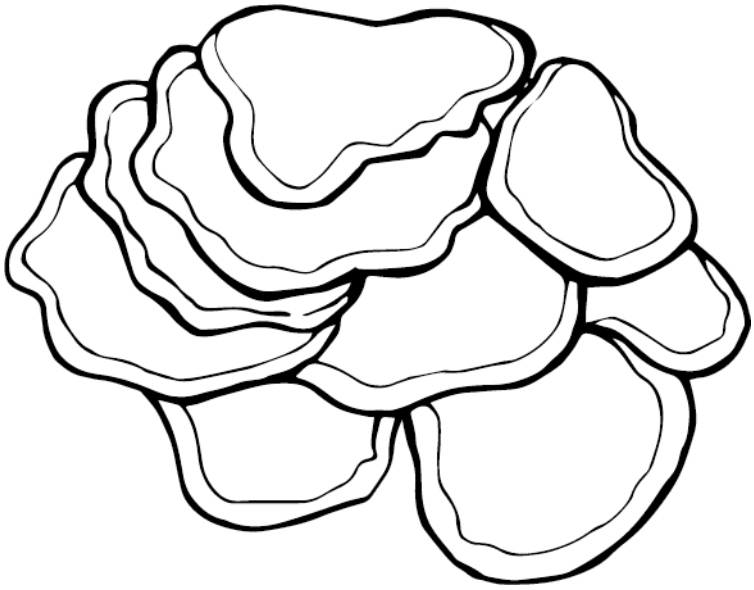

Chapter 3 | **Seasonality affects dinitrogen fixation associated with two common macroalgae from a coral reef in the northern Red Sea**

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3.1 | Abstract

Nitrogen (N) is often a limiting nutrient for primary production in coral reef ecosystems. In this context, dinitrogen (N_2)−fixing prokaryotes (diazotrophs) associated with benthic primary producers can relieve N limitation. Macroalgae are key reef players that are generally able to rapidly take up dissolved inorganic nutrients. They may thus particularly benefit from the activity of associated diazotrophs. With this rationale, this study investigated N_2 fixation activity and net primary production associated with two dominant coral reef macroalgae (the green algal genus *Caulerpa* and the brown algal genus *Lobophora*) during all four seasons in a fringing northern Red Sea reef using the acetylene reduction assay and oxygen production and consumption measurements. Both macroalgae exhibited associated N_2 fixation activity during all seasons with lowest activity in winter and significantly higher activity (one and two orders of magnitude increase for *Lobophora* and *Caulerpa*, respectively) during the nutrient−depleted summer, while net primary production for both macroalgae remained relatively constant over all four seasons. Primary production rates of the macroalgae were comparable to corals from the same area on a yearly average. Conversely, average N_2 fixation rates of both macroalgae were approximately five−fold higher than rates reported for hard corals that were incubated in parallel experiments. These results indicate that macroalgae can capitalize on higher inputs of N from epibiotic diazotrophs, which in turn could prove an ecological advantage when competing for space with corals.

Keywords: Gulf of Aqaba | acetylene reduction | primary production | macroalgae | dinitrogen fixation

A modified version of this chapter has been published in *Marine Ecology Progress Series* 575:69−80. https://doi.org/10.3354/meps12206

3.2 | Introduction

Coral reefs are systems that exhibit notably high primary production despite being surrounded by oligotrophic waters[1]. Due to the scarcity of bioavailable nutrients, benthic organisms such as hard and soft corals have evolved an effective mutualistic symbiosis with single−celled dinoflagellates of the family Symbiodiniaceae[2] (also known as zooxanthellae) for maintaining efficient uptake, recycling and conservation of (in)organic carbon (C), phosphorus (P) and nitrogen (N)[3–5]. In this biogeochemical cycling of nutrients, N is often considered the limiting factor that controls primary productivity (i.e. the fixation of inorganic C through photosynthesis), and is therefore an essential macronutrient for zooxanthellae[6,7]. Uptake of ammonium by the zooxanthellae as a source of inorganic N is preferred over uptake of other forms such as nitrate[8]. Bioavailable N is lost when nitrifying and denitrifying bacteria act together to transform ammonium into dinitrogen (N_2), which most organisms cannot use. However, N_2−fixing prokaryotes, or diazotrophs, are able to restock the bioavailable N pool by converting N_2 into ammonium. Zooxanthellate corals appear to have evolved characteristic associations with diazotrophs[9–11], and recent research indicates that N_2 fixation can provide hard corals with a significant portion of their daily N requirements[12,13]. Thus, the association with diazotrophs may be key to their success in oligotrophic waters.

Besides corals, N_2 fixation activity has been measured for other benthic organisms that represent important functional groups on coral reefs[14], namely macroalgae[15]. Here, diazotrophs inhabit the macroalgae epibiotically[16,17], often in high abundances[18], and may play an important ecological role in (a)biotic interactions with the macroalgal host[19,20]. These macroalgae−diazotroph interactions could provide the macroalgae with otherwise unavailable nutrients and may be essential for their competitive success. Until recently, N_2 fixation in coral reefs did not receive much attention, and data on macroalgae−diazotroph interactions is particularly lacking. To our knowledge only a few studies on macroalgae−associated N_2 fixation activity are available[15,21–24] and some only report N_2 fixation rates from a single isolated species of diazotroph[21,22]. These studies reported N_2 fixation rates for the genera *Sargassum*, *Codium*, *Macrocystis*, *Laurencia*, *Microdictyon*, *Dictyota*, *Padina*, and *Halimeda*. Of these, the latter five were collected from coral reefs.

Anthropogenic stressors will continue to act upon coral reef systems in the future[25], and may well lead to a loss of coral cover (or recruitment) and/or an increase in (macro)algal cover[25–27]. This can cause a progressive shift from coral domination towards (macro)algae domination in what is called a phase shift[28]. In this context, recent studies suggest that ocean acidification and increased sea surface temperatures as well as eutrophication can alter diazotrophic communities associated with hard corals[29,30] as well as their N_2 fixation activity[31,32]. While the coral symbiosis is highly adapted to a low−nutrient regime, and zooxanthellae population densities are effectively controlled by the host by limiting nutrient availability to the algae[33], macroalgae are usually fastgrowing organisms that quickly capitalize on pulses of dissolved nutrients that are otherwise rarely available[34]. In this context, N_2 fixation activity may give macroalgae an additional competitive advantage over corals, especially in a warming ocean[30] and/or in the absence of herbivores due to overfishing[35]. Further studies examining the environmental factors that control the activity of macroalgae−associated diazotrophs are thus important if we want to understand the potential mechanisms underlying coral−algal phase shifts.

N_2 fixation is dependent upon several factors such as light, oxygen (O_2) concentrations, temperature, water flow, and availability of nutrients[14,36–38]. Furthermore, seasonal differences in N_2 fixation have been

observed in hard corals[12], soft corals[11], sponges[39], turf algae[39], coral rock[39], and sediments[40], but not macroalgae. Bednarz et al.[11], Cardini et al.[12] and Rix et al.[39] also suggested that N_2 fixation rates are correlated with productivity of the associated organism, which was especially evident during summer when light intensity and water temperature were highest and nutrient availability lowest. Moreover, Rix et al.[39] found exceptionally high N_2 fixation associated with turf algae compared to values measured in other benthic organisms such as hard corals, likely due to a frequent association of turf algae with cyanobacteria[41]. A high proportion of this fixed N is translocated to the eukaryotic part of the turf algae assemblage (e.g. Rhodophyta, Chlorophyta, and Phaeophyceae) and may thus provide the turf algae with a competitive advantage over other benthic organisms such as hard corals.

The present study extends the current literature by investigating (*i*) N_2 fixation and primary production associated with two common reef macroalgal genera, i.e. *Caulerpa* and *Lobophora*, (*ii*) whether N_2 fixation and primary production are linked, and (*iii*) which environmental factors drive macroalgae primary production and associated N_2 fixation. Finally, we explore (*iv*) how macroalgae−associated N_2 fixation and primary production compare to other key benthic reef organisms and (*v*) what the biogeochemical implications for coral−algal phase shifts could be. N_2 fixation and primary production rates, for both macroalgae and a set of key environmental parameters, were measured during all four seasons of the year 2013 in a northern Red Sea reef. The same methodology (acetylene reduction assay and O_2 measurements, i.e. production and consumption) and normalization parameters (species surface area) were used as in parallel incubation experiments that targeted different benthic reef organisms[11,39] to facilitate comparison of results.

3.3 | Materials and Methods

Study site and environmental monitoring

This study was conducted in 2013 at a fringing coral reef located within a marine reserve in front of the Marine Science Station (MSS) at the northern Gulf of Aqaba, Jordan (29° 27’ N, 34° 58’ E). The area is characterized by strong regional seasonality reflected by substantial variability of environmental key parameters throughout the year[42,43]. In order to examine the effect of seasonality on macroalgae−associated N_2 fixation and primary production, all experiments described below were repeated over four seasonal periods in 2013: February (winter), April (spring), September (summer), and November (autumn). Environmental parameters were continuously recorded at the sampling location at 10 m water depth over the course of the entire study period. This included daily measurements of *in situ* water temperature and light intensity (photosynthetically active radiation [PAR]) using data loggers (Onset HOBO Pendant UA−002−64; temperature accuracy: ±0.53 °C, spectral detection range: 150 to 1200 nm) and a quantum sensor (LI−COR LI−192SA), and weekly collection and processing of seawater samples to quantify inorganic nutrients (dissolved inorganic nitrogen [DIN = ammonium + nitrate + nitrite] and phosphate [DIP]; fluorometrically for ammonium or photometrically for the remaining nutrients), particulate nitrogen (PN), particulate organic carbon (POC), and chlorophyll *a* (chl *a*; fluorometrically) concentrations. A detailed description of the sample and data analysis can be found in Bednarz et al.[40] or Rix et al.[39].

Algae collection and maintenance

Individual fragments (*n* = 8) of two macroalgal genera, *Caulerpa* sp. and *Lobophora* sp. (herein referred to as *Caulerpa* and *Lobophora*, respectively), were collected during each season from the reef slope at 10 m water depth using SCUBA. *Caulerpa* fragments were carefully retrieved with their holdfasts from the sediment, while *Lobophora* leaves were carefully removed from their anchoring rock. All macroalgae were transferred to an outdoor 800 L flow−through aquarium supplied with seawater pumped directly from the reef at 10 m water depth (exchange rate: 4000 L h^{-1}), thereby providing *in situ* water temperature and nutrient levels. Layers of netting were positioned above the tank to adjust PAR levels to those measured *in situ* at 10 m water depth. The algae were allowed to acclimate for approximately 24 h before the incubations described below were carried out in the aquarium under the same environmental conditions.

Quantification of N_2 fixation and primary production

A detailed description of the chamber incubation procedure to quantify algae−associated N_2 fixation and primary production rates, as net photosynthesis (P_n) and dark respiration (R), can be found in Bednarz et al.[11]. Briefly, N_2 fixation was quantified by an adapted acetylene (C_2H_2) reduction technique[44,45]. Macroalgae were incubated under constant stirring (600 rpm) over a full dark−light cycle (24 h) under maximum seasonal PAR (see Table 3.1) in 1 L chambers with the seawater (0.8 L) and headspace (0.2 L) being 10 % C_2H_2−enriched. Gas samples were drawn after 0, 4, 12, 16 and 24 h, and analyzed for ethylene (C_2H_4) concentration using a customized reducing compound photometer (Peak Laboratories, detection limit 100 ppb). P_n rates were quantified via O_2 production measurements over 60 to 90 min between 12:00 and 14:00 h, while R incubations were conducted 1 to 2 h after sunset in complete darkness for 90 to 120 min using a conductivity− and temperature−corrected O_2 optode sensor (MultiLine® IDS 3430, WTW, accuracy: ±0.5 % of measured value). Macroalgae were incubated under identical conditions as for N_2 fixation in individual 1000 mL closed cell respirometric glass chambers. P_n and R were calculated by subtracting the initial O_2 concentration from the end concentration, and C_2H_4 evolution in each incubation chamber was calculated according to Breitbarth et al.[46]. C_2H_4 and O_2 measurements in each incubation chamber were control−corrected (unfiltered seawater) and normalized to incubation time and macroalgal surface area[47].

Statistical analysis

As not all assumptions for standard tests (e.g. ANOVA) were met, the data were analyzed using the non−parametric permutational multivariate analysis of variance (PERMANOVA). To test for differences in parameters (N_2 fixation, P_n and R) between macroalgae genera and seasons, 2−factor PERMANOVAs were performed, based on Bray Curtis similarities of normalized and square−root transformed data. Therefore, Type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations), and pairwise tests were carried out when significant differences occurred. Statistical analyses were carried out using Primer−E version 6 software[48] with the PERMANOVA+ add on[49]. Correlations between N_2 fixation rates, P_n and R rates per season and across all seasons followed by correlation analyses with environmental water parameters across seasons were determined via linear regression using Sigmaplot 12 (Systat software). Unless specified otherwise, significance level was set at $\alpha = 0.05$.

3.4 | Results

Seasonal variations of key environmental factors

All monitored environmental parameters exhibited a strong seasonal pattern with maximum PAR and maximum water temperature during summer, while inorganic nutrients (i.e. DIN and DIP) and chl *a* concentration were lowest during summer (Table 3.1). Conversely, winter and spring displayed the most distinct environmental parameters compared to summer, followed by autumn (Table 3.1).

Table 3.1 | Summary of key environmental water parameters monitored at 10 m water depth during four seasons.

Environmental variable	Winter	Spring	Summer	Autumn
PAR (µmol m^{-2} s^{-1})	180 ± 15	257 ± 9	317 ± 17	159 ± 18
Temperature (°C)	23.0 ± 0.1	22.8 ± 0.1	27.5 ± 0.2	25.2 ± 0.2
DIN (µM)	1.03 ± 0.02	1.02 ± 0.11	0.20 ± 0.04	0.43 ± 0.08
Ammonium (µM)	0.32 ± 0.04	0.46 ± 0.03	0.14 ± 0.03	0.28 ± 0.06
Nitrate (µM)	0.34 ± 0.03	0.44 ± 0.04	0.04 ± 0.01	0.13 ± 0.05
Nitrite (µM)	0.37 ± 0.06	0.12 ± 0.04	0.02 ± 0.01	0.02 ± 0.01
DIP (µM)	0.11 ± 0.01	0.10 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
DIN:DIP	9.59 ± 1.09	10.21 ± 0.43	5.31 ± 3.40	11.25 ± 2.22
POM (µM)	7.18 ± 0.70	11.52 ± 1.48	8.92 ± 1.23	9.68 ± 0.49
POC:PN	7.34 ± 0.57	8.18 ± 0.59	8.34 ± 0.44	10.20 ± 0.51
Chl *a* (µg L^{-1})	0.21 ± 0.01	0.22 ± 0.02	0.10 ± 0.01	0.19 ± 0.02

PAR = photosynthetically active radiation, DIN = dissolved inorganic nitrogen, DIP = dissolved inorganic phosphate, POM (POC+PN) = particulate organic matter, POC = particulate organic carbon, PN = particulate nitrogen. Values are given as means ± SE (n = 4).

N_2 fixation activity associated with Lobophora *and* Caulerpa

Both macroalgae exhibited associated N_2 fixation during all four seasons indicated by high C_2H_4 evolution rates in algae−containing incubation chambers, while rates in the seawater controls were negligible. Macroalgae−associated N_2 fixation activity, expressed per algal surface area and averaged across the four seasons, resulted in similar values, i.e. 0.89 ± 0.19 and 1.07 ± 0.24 nmol C_2H_4 cm^{-2} h^{-1} for *Lobophora* and *Caulerpa*, respectively. In a seasonal comparison, *Caulerpa* revealed maximum N_2 fixation rates during spring and summer and lowest rates in winter, followed by autumn (Figure 3.1A). In contrast, *Lobophora* showed significantly increased N_2 fixation rates during summer ($p < 0.001$), while the lowest rates were measured in winter followed by spring and autumn (Figure 3.1A).

Primary production of Lobophora *and* Caulerpa

P_n and R differed significantly between the two investigated macroalgae genera ($p < 0.001$). *Caulerpa* displayed higher rates than *Lobophora*, averaging 0.900 ± 0.059 and 0.300 ± 0.015 µmol O_2 cm^{-2} h^{-1} for P_n and 0.096 ± 0.011 and 0.067 ± 0.015 µmol O_2 cm^{-2} h^{-1} for R, respectively, across all seasons. P_n was similar in winter, spring, and summer for both macroalgae, but decreased significantly for both genera from summer to autumn (Figure 3.1B). P_n was similar for autumn and winter in *Caulerpa*, but similar for autumn and spring in *Lobophora* (Figure 3.1B). R was significantly higher in *Caulerpa* compared to *Lobophora* in all seasons except autumn (Figure 3.1C) but followed roughly the same fluctuating pattern throughout the year for both macroalgae.

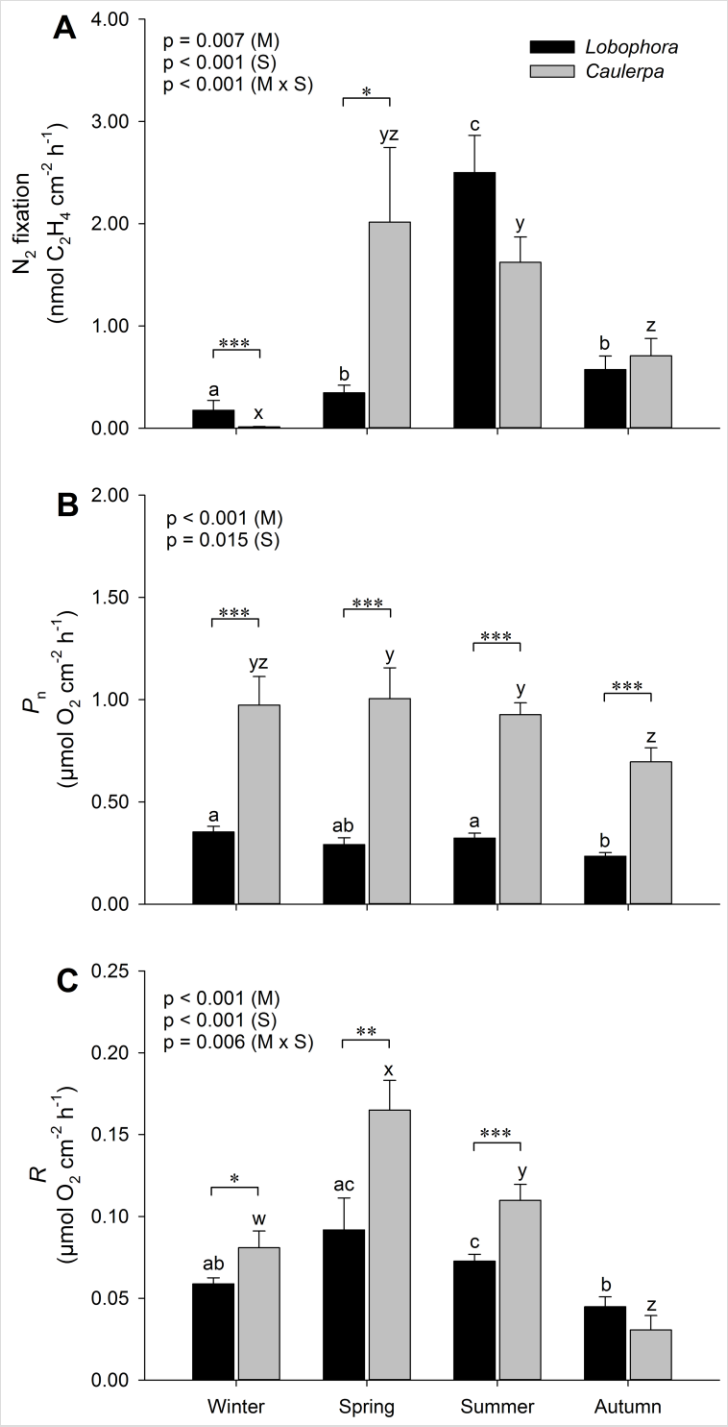

Figure 3.1 | **(A)** N_2 fixation, **(B)** net photosynthesis (P_n) and **(C)** dark respiration (R) associated with the two macroalgae (*Caulerpa* and *Lobophora*) measured during four different seasons (winter, spring, summer, autumn). Values are given as mean ± SE (n = 8). The significant factor (M = macroalgae, S = season, M x S = interaction) is displayed for each parameter. *indicates significant differences between the macroalgae during each season (* p < 0.05, ** p < 0.01, *** p < 0.001) and different letters indicate significant differences within each macroalga per parameter between the seasons (*Lobophora*: a, b and c; *Caulerpa*: w, x, y, z), based on pair−wise PERMANOVA analysis.

Relationships of metabolic rates and environmental factors

Linear regression analyses revealed a negative relationship between N_2 fixation and P_n for *Lobophora* during autumn (F = 23.85, r^2 = 0.799, p = 0.003) (Figure 3.2D), while a positive relationship was found in the same season for *Caulerpa* (F = 8.25, r^2 = 0.579, p = 0.03) (Figure 3.2H). No relationships could be established for winter, spring or summer (Figure 3.2). Also, N_2 fixation rates and R correlated positively for *Caulerpa* during spring (F = 7.81, r^2 = 0.566, p = 0.03; data not shown), while no other correlations for R were found. A positive relationship was found between N_2 fixation rates and P_n for *Caulerpa* across all seasons, while no such relationship was found for *Lobophora*. However, the positive relationship was mainly due to two high values, so that no relationship was found when these two values were excluded. In response to environmental parameters, positive relationships were revealed for *Lobophora*–associated N_2 fixation for temperature and PAR, while negative relationships were found for DIN and DIP (Table 3.2). For *Caulerpa*–associated N_2 fixation, linear regression analysis revealed a significant positive relationship only with PAR (Table 3.2). No relationships were found for P_n with any environmental parameters for both macroalgae (Table 3.2). In addition, a positive relationship was found for R and DIP availability in *Caulerpa*, while no relationships were found for R in *Lobophora* (Table 3.2).

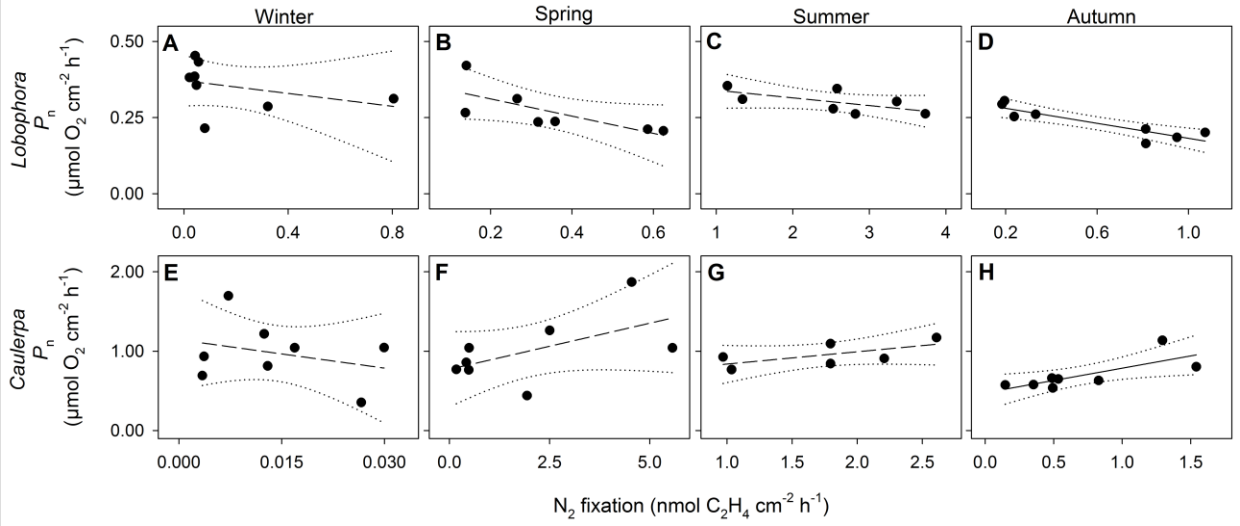

Figure 3.2 | Seasonal relationships between N_2 fixation and net primary productivity (P_n) for *Lobophora* for **(A)** winter, **(B)** spring, **(C)** summer, and **(D)** autumn, and *Caulerpa* for **(E)** winter, **(F)** spring, **(G)** summer, and **(H)** autumn (n = 6 to 8). Best–fit linear regression lines: (——) significant relationship was established; (– – –) not significant; (·····) ± 95% confidence intervals. Note the different values of all x–axes.

3.5 | Discussion

Macroalgae–associated N_2 fixation and primary production

Previous studies reported macroalgae–diazotroph interactions for pelagic and benthic red, brown, and green macroalgae from temperate seas and tropical coral reefs as a ubiquitous and important physiological symbiosis[21–23]. In some of these studies, a single diazotroph species was isolated and tested for N_2 fixation rates[21,22]. Here, N_2 fixation rates and primary production associated with the whole consortium of two

coral reef macroalgal holobionts are reported, i.e. the eukaryotic host and its associated diazotrophic community.

The present study found no differences in annual averaged N_2 fixation between the two macroalgae, i.e. *Caulerpa* and *Lobophora*, suggesting similar yearly activity of the diazotrophic community. To the best of our knowledge, this is the first study to normalize N_2 fixation activity to macroalgal surface area, whereas previous studies normalized to dry weight of the macroalgae[21,24], making comparisons challenging. However, when comparing dry weight normalized N_2 fixation rates of brown and green algae, Capone et al.[15] also found similar N_2 fixation rates between the green alga *Halimeda* and brown alga *Padina*, while in the same study, they found that the green alga *Microdictyon* and brown alga *Dictyota* were also similar to each other but had five to ten times higher rates. Thus, rates observed here may not necessarily be representative for other green and brown algae found in the northern Red Sea.

In contrast, primary production differed between the macroalgal genera. This may be due to different preferred light regimes necessary for green and brown algae since they have evolved associations with different pigments that allow for optimal photosynthesis at different depths[50] and thus caused by a natural physiological boundary. Furthermore, morphological differences between both genera could also explain these differences. Algae such as *Caulerpa* with a more complex or filamentous morphology have improved uptake of nutrients due to their surface area:volume ratio and can therefore exhibit higher primary production rates[51,52]. Furthermore, *Lobophora* may favor protection from herbivores over higher primary production. High uptake of N and subsequent high primary production may come at the expense of anti−herbivory strategies as high N uptake is negatively correlated with the production of phlorotannins[53].

Table 3.2 | Linear regression analysis (r^2 values) for N_2 fixation, net photosynthesis and dark respiration rates of two macroalgae and four different environmental water parameters.

	PAR	Temperature	DIN	DIP
Caulerpa				
N_2 fixation	**0.216***	0.015	0.011	0.011
P_n	0.041	0.024	0.051	0.080
R		0.059	0.121	**0.167***
Lobophora				
N_2 fixation	**0.472*****	**0.656*****	*0.517****	*0.347****
P_n	0.034	0.005	0.031	0.085
R		0.013	0.031	0.045

PAR = photosynthetically active radiation, DIN = dissolved inorganic nitrogen, DIP = dissolved inorganic phosphate, P_n = net photosynthesis, R = dark respiration. Bold characters indicate significant positive relationships, and italicized characters indicate significant negative relationships. *$p < 0.05$, ***$p < 0.001$.

Relationship between N_2 fixation and primary production

Related studies using similar methods at the same location in the Red Sea found positive correlations between N_2 fixation and parameters of primary production (i.e. P_n, gross photosynthesis and/or R) of the associated benthic organism[11,12,39]. In this study, in a seasonal comparison, both N_2 fixation and P_n declined for both macroalgae during the transition from summer to autumn. Indeed, linear regression confirmed a relationship between the two processes during autumn for both macroalgae. Although the energy−demanding process of N_2 fixation is highly dependent on photosynthesis as an energy source[54], the correlation in *Lobophora* was negative. This may suggest, for this algal species, a diazotrophic

community dominated by non−heterocystous diazotrophs that cannot fix N_2 when oxygen is produced as a byproduct of photosynthesis[21,55]. In contrast, *Caulerpa* revealed a positive relationship between N_2 fixation and P_n, suggesting the presence of either heterocystous cyanobacteria, or diazotrophs with other mechanisms in place to protect the nitrogenase enzyme from oxygen inhibition. Despite the strong decline of N_2 fixation rates for *Caulerpa* from summer to autumn, the significant drop in P_n was not as strong as expected. This may be due to ambient DIN concentrations increasing during autumn. The DIN: DIP ratio remained below the 16:1 Redfield ratio, indicating that N remained the limiting nutrient in all seasons. This suggests that the combined concentrations of ambient DIN and bioavailable N provided by the diazotrophic community are responsible for maintaining fairly stable primary production rates in *Caulerpa*[56,57]. In addition, *R* correlated with N_2 fixation rates for *Caulerpa* during spring when N_2 fixation rates were high, while no correlations were found for *Lobophora*. For *Caulerpa*, *R* declined significantly from spring to summer while N_2 fixation rates remained similar. In contrast, *R* for *Lobophora* remained stable from spring to summer, while N_2 fixation rates increased dramatically in summer. This suggests either differences in diazotrophic activity or community structure between seasons for both macroalgae.

Seasonal patterns in N_2 fixation and primary production

Macroalgae−associated N_2 fixation rates revealed high sensitivity to seasonally changing environmental conditions, whereas the net primary productivity of the macroalgae exhibited only very minimal seasonal change. The overall N_2 fixation rate pattern of *Lobophora* revealed highest N_2 fixation rates during summer when PAR and ambient water temperature were highest and nutrient availability (DIN and DIP) lowest. This was further substantiated by linear regression as a correlation was found for each of these parameters. Head & Carpenter[22] also found a positive correlation between N_2 fixation rates and PAR in the green macroalga *Codium fragile*. They also reported reduced primary production and N_2 fixation rates in shaded conditions. Here, N_2 fixation rates were positively correlated with PAR, while no correlation was found for primary production. Thus, PAR may have been saturating throughout the year for primary production[58]. Substantially lower rates of N_2 fixation were found during winter, spring and autumn when nutrient availability was higher. Indeed, N_2 fixation rates are likely to be affected by availability of DIN in particular[22,38]. While our results report relationships on the scale of a single genus, the same patterns can be found on the community level. Overall community rates of benthic N_2 fixation appear to be strongly affected by seasonality, while primary production remains fairly similar[59]. Moreover, this characteristic is not limited to salt water systems, as this has also been observed in oligotrophic Arctic freshwater lakes[60]. Interestingly, like in the present study, Gettel et al.[60] also found that primary production remained fairly similar under different N_2 fixation rates. Thus, it is most likely that N_2 fixation rates in *Lobophora* used in this study were primarily regulated by nutrient availability and temperature, which can also have a positive effect on the nitrogenase enzyme[14].

The observed pattern of N_2 fixation for *Caulerpa* was similar to *Lobophora* with the exception of the spring season. In *Caulerpa*, highest N_2 fixation rates were found in spring and in summer. Moreover, like *Lobophora*, a positive correlation between N_2 fixation and PAR was found for *Caulerpa*. However, N_2 fixation rates during spring were not significantly different from summer, when PAR was highest, and autumn, when PAR was at its lowest. It is likely that the high variability during spring confounded the data, causing a lack of correlation for all other parameters. This may have been caused by physiological differences between the sampled tissues of the macroalgal genotypes, due to age of the blades[61], or the

presence/absence of heterocystous diazotrophs. Moreover, this high variability may also be explained by inhibition of nitrogenase activity due to the presence of a higher ammonium concentration[37], while at the same time nitrogenase activity may be increased due to higher water temperatures and irradiance[14]. This observed pattern disparity between *Caulerpa* and *Lobophora* suggests that diazotrophic communities may differ interspecifically while the high variability in spring for *Caulerpa* even suggests large intraspecific differences[62].

The seasonal pattern of N_2 fixation rates observed for *Lobophora* show a striking similarity to the one observed for coral rock investigated in parallel[39], including the apparent discrepancy during spring. Interestingly, the pattern observed for *Caulerpa* was similar to the one observed for carbonate sand investigated in parallel[40]. Both macroalgae were sampled from these respective substrates. Linear regression analysis, using mean N_2 fixation rates (± SE) per season from coral rock (data taken from Rix et al.[39]) and carbonate sand (data taken from Bednarz et al.[40]) compared to N_2 fixation rates for both macroalgae measured in this study, shows a clear significant correlation ($r^2 = 0.8748$; $p < 0.001$) (Figure 3.3). This strongly suggests that the diazotrophic community structures of both macroalgae are similar to their associated substrate[63].

The apparent (synergistic) links of temperature, light, and nutrient availability with N_2 fixation rates reported here highlight the complexity of eukaryote−diazotroph (macroalgal holobiont) interactions with their environment, even within a single functional group (i.e. macroalgae) or a single species/genus. Identification, relative abundance, and activity measurements of the total microbial and diazotrophic community, and also for the macroalgae−associated substrates, may shed some light on observed N_2 fixation patterns during all four seasons.

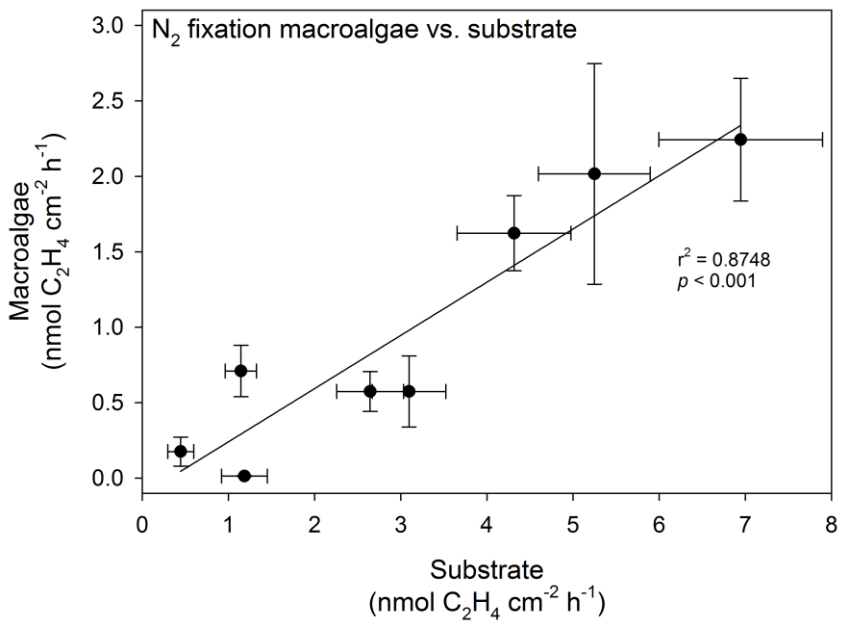

Figure 3.3 | Linear regression analysis of N_2 fixation rates of macroalgae (*Lobophora* and *Caulerpa*) and their associated substrate (coral rock and carbonate sand) throughout the year. Substrate data combines coral rock data from Rix et al.[39] and carbonate sand data from Bednarz et al.[40]. Values are given as mean ± SE.

Comparison with parallel investigated organisms

The yearly macroalgae–associated N_2 fixation rates reported in the present study (9.56 ± 3.95 and 7.81 ± 4.03 µmol C_2H_4 cm^{-2} yr^{-1} for *Caulerpa* and *Lobophora*, respectively) were higher compared to hard and soft corals investigated in parallel (Table 3.3). N_2 fixation rates were lowest for Xeniidae at 0.18 ± 0.12 µmol C_2H_4 cm^{-2} yr^{-1} and highest for *Goniastrea* sp. (now *Coelastrea* sp.[64]) at 2.14 ± 1.03 µmol C_2H_4 cm^{-2} yr^{-1} (Table 3.3), resulting in 9 and up to 53 times higher N_2 fixation rates in the investigated macroalgae. These differences could be attributed to abundances, community structure, and/or metabolism of diazotrophs associated with each organism[62]. Overall, primary production rates of all benthic organisms compared by this study were relatively similar (Table 3.3). However, in corals, large quantities of photosynthetic products (dissolved organic carbon [DOC]) are transferred to the coral host[65,66]. Thus, less DOC may be available for the coral–associated microbial community. Macroalgae can release considerably higher proportions of DOC compared to corals[67], which could be particularly beneficial as an energy source for (epibiotic) heterotrophic microbes. Furthermore, because of its composition, DOC released by macroalgae can cause considerably higher growth of microbes compared to DOC released by corals[68]. At the same time, epibiotic diazotrophs on macroalgae are usually exposed to good light conditions that may provide an optimal light regime for photosynthetic diazotrophs, including cyanobacteria[62]. Thus, we suggest that macroalgae are a more favorable host for diazotrophs compared to corals, providing optimal conditions for both heterotrophic (algae–derived DOC as energy source) and autotrophic (light as energy source) species.

Table 3.3 | Comparison of N_2 fixation activity and net primary production associated with different benthic coral reef organisms from the northern Red Sea.

Reef organism	N_2 fixation activity (µmol C_2H_4 cm^{-2} yr^{-1})	Net primary production (mmol O_2 cm^{-2} yr^{-1})	Reference
Green alga *Caulerpa* sp.	9.56 ± 3.95	7.88 ± 1.44	This study
Brown alga *Lobophora* sp.	7.81 ± 4.03	2.63 ± 0.37	
Hard coral *Acropora* sp.	0.91 ± 0.73	5.03 ± 0.66	Cardini et al.[12]
Hard coral *Pocillopora* sp.	1.07 ± 0.44	4.54 ± 0.81	
Hard coral *Stylophora* sp.	1.38 ± 0.67	4.21 ± 0.93	
Hard coral *Goniastrea* sp.	2.14 ± 1.03	5.38 ± 0.74	
Soft coral Xeniidae	0.18 ± 0.12	3.43 ± 0.50	Bednarz et al.[11]
Soft coral *Sarcophyton* sp.	0.52 ± 0.24	1.86 ± 0.71	
Sponge *Mycale* sp.	1.72 ± 1.07	−2.10 ± 0.65	Rix et al.[39]
Turf algae	40.09 ± 19.35	4.59 ± 0.91	

The mean value of each season was used to calculate the annual average. Values are given as mean ± SE (n = 4).

N_2 fixation rates presented in this study were lower compared to turf algae investigated in parallel, which had N_2 fixation rates of 40.09 ± 19.35 µmol C_2H_4 cm^{-2} yr^{-1}, while rates for the macroalgae were up to 5 times lower (Table 3.3). This could be explained by (a combination of) two reasons. Firstly, these differences could be attributed to turf algae assemblage structure and characteristics, such as high turnover and opportunistic growth dynamics[69,70]. Given these life–history traits, it is not unlikely that the diazotrophic part of the turf algae assemblage displays similar characteristics, i.e. fast growth and high activity to provide bioavailable N to facilitate growth of the assemblage. Secondly, even though P_n rates found in this study are relatively similar to those in turf algae, release of organic matter in the form of DOC

is considerably higher in turf algae (compared to macroalgae) and also subject to seasonality[71]. Surprisingly, Haas et al.[71] reported lowest DOC release rates during summer, while the N_2 fixation rates reported by Rix et al.[39] were highest during summer. Besides eukaryotic algae, turf algae assemblages may consist of high numbers of filamentous cyanobacteria[72]. However, high abundances of heterotrophs can be found in these cyanobacterial mats[73]. Thus, instead of being released in the water column, it is likely that released DOC is rapidly utilized by the microbial community (and thus not measurable), providing energy for N_2 fixation.

Implications for coral–algal phase shifts

With sea surface temperatures expected to exceed coral temperature thresholds more often in the future[25], following the potential subsequent mass mortality of corals due to bleaching, phase shifts from coral– to algae–dominated reefs are more likely to occur. Moreover, recent research has revealed that benthic algae, such as *Lobophora*, can rapidly utilize excess nutrients from terrestrial run–off and thrive under these conditions[34], while corals, depending on the type of eutrophication, possess reduced resilience[74,75]. Thus, eutrophication can result in the loss of coral cover and give rise to potential spaces for (macro)algae to grow. Also, following disturbances, (macro)algae can rapidly colonize new territory[76], possibly facilitated by higher N_2 fixation rates compared to e.g. hard corals. *Lobophora* in particular can occupy substrates otherwise available for coral recruits[77] and can cause coral mortality by shading[78]. This study and that of Rix et al.[39] reveal that N_2 fixation rates in macroalgae, as well as turf algae, were significantly higher than those measured in corals investigated in parallel, while their primary production was similar (Table 3.3). The high variability of N_2 fixation rates observed in turf algae assemblages[39] makes it difficult to determine whether they are able to outcompete the macroalgae investigated in the present study. Thus, a qualified statement on competition between turf and macroalgae cannot be made.

N_2 fixation rates appeared to be affected by DIN with lower diazotrophic activity under less oligotrophic conditions. These findings indicate that warming–induced phase shifts from corals to (macro) algae could result in increased diazotrophic–driven import of N in tropical shallow coastal environments. In perspective, this may suggest that ocean warming and a subsequent increase in the input of fixed N by diazotrophs could result in phase shifts even in the absence of anthropogenic eutrophication. In addition, this input of fixed N could potentially facilitate an increase in DOC release by turf algae[79], creating a positive feedback loop that can be detrimental to coral health status[30,71,80]. Although increased ammonium concentrations downregulate N_2 fixation activity during and after a phase shift, diazotrophs may act as an important trigger for changing the ecosystem.

In conclusion, while both climate change–related stressors and eutrophication can cause phase shifts, the results presented here indicate that the type of disturbance is likely to influence N_2 fixation rates differently. Whereas increased sea surface temperatures (partly) correlated with increased N_2 fixation rates, so did declining ambient DIN availability. The apparent role of DIN in the N_2 fixation rates reported here and in related literature suggest that input of N from allochthonous sources may cause N_2 fixation to become an obsolete biological mechanism for producing bioavailable N, and also for macroalgae during phase shifts. Our study further suggests that ocean warming may be accompanied by higher diazotroph activity associated with macroalgae, but only while oligotrophic conditions persist.

3.6 | Acknowledgments

We thank F. Al−Horani and S. Basuoni for field−work assistance and logistical support, F. Dini−Andreote for statistical support, and D. Dasbach, A. Dumeier, N. Rädecker, and D. Peterke for their help with sample analyses. This work was funded by the German Research Foundation (grant numbers Wi 2677/6−1 and Wi 2677/9−1to C.W.) and a PhD stipend from the Evangelisches Studienwerk Villigst e.V. to V.N.B.

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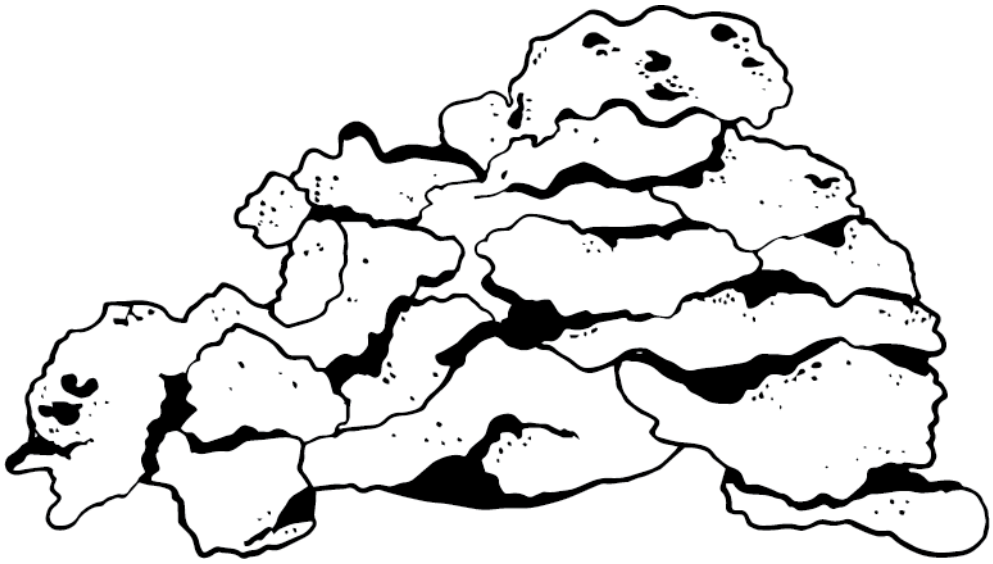
Chapter 4



Chapter 4 | **Relative diazotroph abundance in symbiotic Red Sea corals decreases with water depth**

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4.1 | Abstract

Microbial dinitrogen (N_2) fixation (diazotrophy) is a trait critical for coral holobiont functioning. The contribution of N_2 fixation to holobiont nitrogen (N) supply likely depends on the ecological niche of the coral holobiont. Consequently, coral–associated diazotroph communities may exhibit distinct activity patterns across a water depth gradient. We thus compared relative abundances of diazotrophs in the tissues of two common hard coral species, *Podabacia* sp. and *Pachyseris speciosa*, along their water depth distribution (10–30 m and 30–50 m, respectively) in the Central Red Sea. The relative gene copy numbers of the *nifH* gene (i.e., referenced against the eubacterial 16S rRNA gene), as a proxy for N_2 fixation potential, were assessed via quantitative PCR. We hypothesized that relative *nifH* gene copy numbers would decrease with water depth, assuming a related shift from autotrophy to heterotrophy. Findings confirmed this hypothesis and revealed that *nifH* gene abundances for both corals decreased by ~97 % and ~90 % from the shallowest to the deepest collection site. However, this result was not significant for *Pachyseris speciosa* due to high biological variability. The observed decrease in *nifH* gene abundances may be explained by the relative increase in heterotrophy of the coral animal at increasing water depths. Our results underline the importance of interpreting microbial functions and associated nutrient cycling processes within the holobiont in relation to water depth range reflecting steep environmental gradients.

Keywords: Coral reefs | diazotrophy | heterotrophy | autotrophy | nitrogen fixation | depth gradient

A modified version of this chapter has been published in *Frontiers in Marine Science* 6:372.
https://doi.org/10.3389/fmars.2019.00372

4.2 | Introduction

Scleractinian corals are associated with a diverse microbial community. This microbiome is comprised of dinoflagellate algae of the family Symbiodiniaceae[1] and a multitude of other eukaryotic and prokaryotic microbes that together form a meta–organism, the coral holobiont[2]. Coral–associated microbiomes are host–specific, and provide key functions to the holobiont, such as provision and cycling of essential nutrients (among others)[2–6]. Among these, diazotrophy or biological fixation of dinitrogen (N_2), a functional trait associated with diverse Bacteria and Archaea, i.e., diazotrophs, is associated with a broad range of scleractinian corals[7–13]. Diazotrophy, along with other nitrogen (N) cycling pathways, was proposed central to the exceptional adaptation of scleractinian corals to oligotrophic environments[5,7,8,14]. Indeed, coral–associated N_2 fixation provides an important source of N for Symbiodiniaceae[8,15–17], thereby helping to sustain holobiont productivity in shallow water corals when nutrients are scarce[8,15,17].

Microbial N_2 fixation is catalyzed by the nitrogenase enzyme complex, the activity of which is affected by its abiotic environment. Elevated levels of dissolved oxygen (O_2) for instance can irreversibly deactivate dinitrogenase reductase, one of the two subunits of the enzyme complex[18], thereby hampering N_2 fixation activity. Further, the enzymatic activity of the nitrogenase complex increases with temperature[18] and is hypothesized to decrease with increased environmental availability of bioavailable N (commonly referred to as "ammonia switch–off"[19]). As O_2 availability within the coral tissues can exhibit considerable diurnal variation due to high rates of photosynthesis by Symbiodiniaceae during the day[20], irradiance may be a critical driver of coral–associated N_2 fixation[17]. These environmental parameters are subject to temporal and spatial change on a coral reef. Thus, to meet the metabolic carbon (C) and N requirements of the holobiont, scleractinian corals exhibit shifts in resource partitioning and may display variable levels of heterotrophy[17,21,22].

Acquisition of N through N_2 fixation in the coral holobiont is an energetically costly process that is likely fueled by photosynthetically fixed C (photosynthate) of the Symbiodiniaceae[15]. Given the attenuation of solar irradiance with depth in the water column[23–25], autotrophic potential of the coral holobiont potentially decreases with depth as well, making the holobiont rely more on heterotrophy to fulfill its energy requirements[26,27]. As heterotrophic food sources have a higher N content than photosynthate provided by Symbiodiniaceae, the N demand of the coral holobiont may decrease with increasing heterotrophy[28]. In this light, Pogoreutz et al.[12] showed a negative correlation of N_2 fixation activity with heterotrophic potential of scleractinian corals by using relative *nifH* gene copy numbers as a proxy for N_2 fixation potential. Thus, we hypothesized that corals at the shallower end compared to their deeper vertical distribution ranges will exhibit differential relative N_2 fixation activity, i.e., lower relative N_2 fixation activity with increasing depths. In order to test this hypothesis, we (*i*) assessed diazotroph abundances in two Red Sea corals, the mushroom coral *Podabacia* sp. (Fungiidae) and *Pachyseris speciosa* (Agariciidae), along a depth gradient spanning 10–50 m, and (*ii*) assessed the correlation of environmental parameters along the corals' depth gradient with the observed depth–dependent patterns of diazotroph abundance. To assess the relative abundance of diazotrophs in the coral microbiome, relative *nifH* gene copy numbers were obtained via quantitative PCR of the *nifH* gene normalized to the 16S rRNA gene[12,13].

4.3 | Materials and Methods

Coral collection

Two species of scleractinian coral, *Podabacia* sp. and *Pachyseris speciosa*, were sampled using SCUBA and technical diving at the midshore reef Al Fahal (N22°18'19.98'', E38°57'46.08'') in the Saudi Arabian central Red Sea in November 2012[29]. The Saudi Arabian Coastguard Authority issued sailing permits to the site that include coral collection. Both corals are listed as "least concern" according to the IUCN Red List of Threatened Species[30,31]. One fragment from the central upward–facing surface per colony (n = 3–5) of both species were sampled in 10 m intervals from 10 to 50 m water depth. The depths at which the corals were sampled reflects the main distribution of each coral species at our sampling site (see sample distribution in Table 4.1).

Table 4.1 | Distribution and number of samples used to assess diazotroph abundances associated with the tissues of two species of reef–building corals along a water depth gradient in the central Red Sea.

Water depth (m)	10	20	30	40	50
Podabacia sp. (n)	3	3	3	–	–
Pachyseris speciosa (n)	–	–	5	3	4

Quantification of diazotroph communities

Coral tissue was removed from snap–frozen fragments with ice cold 4 % NaCl solution using a standard airgun and an airbrush (Airbrush–starter–set, Conrad Electronic SE, Germany). Tissue slurries were homogenized and stored at −20 °C until further processing. DNA was extracted from coral tissue slurries using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer's instructions. For DNA extraction, 100 µL aliquots of each tissue slurry sample were used in 300 µL AP−1 buffer. Extracted DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, United States) and adjusted to a DNA concentration of 5 ng μL^{-1}.

To determine coral tissue–associated diazotroph abundances (as a proxy for N_2 fixation potential[12]), relative gene copy numbers were quantified for the prokaryotic *nifH* gene (as the target gene) and the bacterial 16S rRNA (as a reference gene and a proxy for the total bacterial community) as reported previously[13]. The 16S rRNA gene was selected as a reference gene in the present study as it exhibited stable relative gene copy numbers in coral tissues across the entire water depth distribution range (see Supplementary Figure S4.1). Relative quantification was achieved with quantitative PCR (qPCR) in triplicate reactions with the Platinum SYBR Green qPCR SuperMix–UDG (Invitrogen, United States) according to manufacturer's instructions. Amplifications were performed with 5 µL SuperMix, 0.2 µL ROX reference dye, 0.2 µL of each 10 µM primer, 1 µL of input DNA template, and RNAse–free water to adjust the reaction volume to 10 µL. To amplify the bacterial 16S rRNA gene, the primers 781F (5'−TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG−3') and 1061R (5'−GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA−3') were used[32]. This primer pair includes the region V6 and covers the position 781 to 1,060. For amplification of the *nifH* gene, the degenerate primer pair F2 (5'−TGYGAYCCIAAIGCIGA −3') and R6 (5'−TCIGGIGARATGATGGC−3') was used[33]. This primer pair covers the position 115 to 473. The qPCR was run under the following thermal profile: 2 min at 50 °C, 1 min at 94 °C, followed by 50 cycles of 30 s at 94 °C, 1 min at 51 °C, 1 min at 72 °C, and an extension cycle of 1 min at 72 °C[12]. The specificity of the 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 for the 16S rRNA and *nifH* gene). 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 for all depths and both species as $2^{(-\Delta\Delta Ct)}$ using samples from the deepest sampling location of the respective species as a reference. Different depths at which both corals were collected (overlap only at 30 m) precluded direct comparisons of relative *nifH* gene abundances between both species.

Environmental parameters

Environmental parameters at the collection site were collected on 3 days (within 10 days of each other) around noon in September 2012. All measuring procedures and environmental data were previously described and published in Ziegler et al.[29]. Briefly, a conductivity–temperature–depth recorder (CTD; SBE 16plusV2, Seabird Electronics, United States) was deployed to 50 m depth in close proximity to the sampling location measuring water temperature, photosynthetically active radiation (PAR), O_2 saturation, salinity, turbidity, and chlorophyll concentration approximately every 10 m. Measurements at each depth lasted approximately 1 min.

Statistical analyses

Data were analyzed using non–parametric permutational multivariate analysis of variance (PERMANOVA) using PRIMER–E version 6 software[34] with the PERMANOVA+ add on[35]. To test for differences in relative *nifH* gene copy numbers per species and individual environmental parameters across depths, 1–factorial PERMANOVAs were performed, based on Euclidean distances of normalized and/or square–root transformed data. Type I (sequential) sum of squares was used with unrestricted permutation of raw data (999 permutations) and PERMANOVA pairwise tests with parallel Monte Carlo tests were carried out when significant differences occurred.

To identify the environmental parameter that "best explains" the multivariate physiological pattern of the coral samples, a biota–environmental matching (BIOENV) routine was computed with 999 permutations based on Euclidean distances. This analysis maximizes Spearman rank correlations between the resemblance matrices through permutation of all trial variables. Salinity and turbidity were omitted from the analysis due to their small–scale differences along the depth gradient. Input data were normalized prior to analysis by subtracting the variable's mean from each value and dividing it by the standard deviation using PRIMER normalization option. Finally, a correlation with depth for Symbiodiniaceae cell density of the investigated coral species (data obtained from Ziegler et al.[29]) and relative *nifH* gene copy numbers was determined via linear regression using SigmaPlot 12 (Systat software).

4.4 | Results

Coral diazotroph abundances along a depth gradient

The qPCR results confirmed the presence of the *nifH* gene in the microbial communities associated with both investigated Red Sea coral species and across the entire distribution range of sampling depths (Figure 4.1). Despite belonging to two different coral families (Fungiidae and Agariciidae) and exhibiting differences in water depth distribution range, the two coral species exhibited a similar pattern: relative *nifH* gene copy numbers for each coral species were highest at the two shallowest water depths

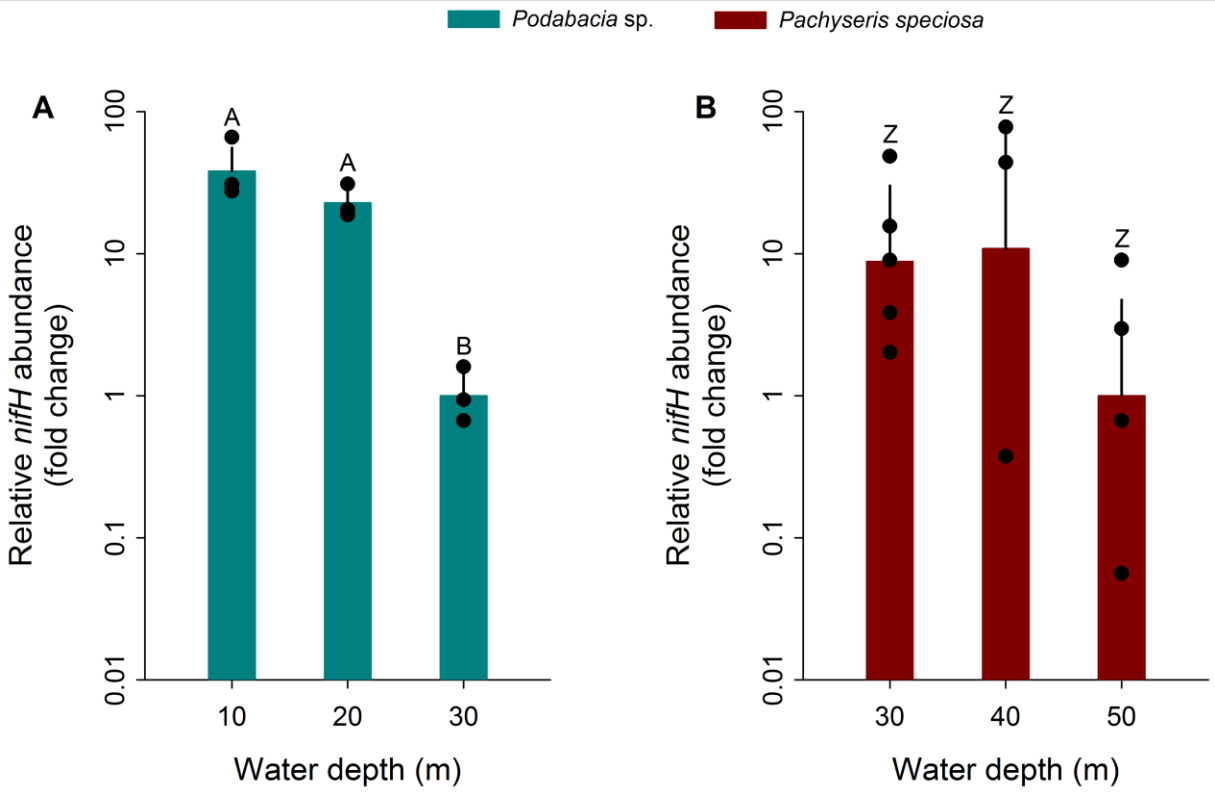

Figure 4.1 | Fold changes in relative *nifH* gene copy numbers normalized to 16S rRNA gene copy numbers in the tissues of two Red Sea scleractinian corals **(A)** *Podabacia* sp. and **(B)** *Pachyseris speciosa* (n = 3–5) depending on water depth. Fold changes were calculated in relation to the greatest collection depth of the respective species; error bars indicate upper confidence intervals (+ 1 SE). Black circles represent data points. Different letters above bars indicate significant differences within species ($p < 0.01$). As both corals were collected at different depths (limited overlap only at 30 m), direct comparisons of relative *nifH* gene abundances between both species were precluded.

Table 4.2 | Summary of depth effects on relative *nifH* gene copy numbers associated with the tissues of two Red Sea coral species and for environmental factors based on permutational multivariate analysis of variance (PERMANOVA).

Parameter	Effect	df	Pseudo–F	*p*
Relative *nifH* gene copy numbers *Podabacia* sp.	Depth	2	53.2	**0.001**
Relative *nifH* gene copy numbers *Pachyseris speciosa*	Depth	2	1.7	0.230
Temperature	Depth	5	441.7	**0.001**
Photosynthetic Active Radiation (PAR)	Depth	5	343.2	**0.001**
Dissolved oxygen (O_2)	Depth	5	19.3	**0.001**
Salinity	Depth	5	15.9	**0.001**
Turbidity	Depth	5	0.9	0.457
Chlorophyll concentration	Depth	5	29.0	**0.001**

and decreased at the greatest water depth (Figure 4.1). Significant differences with water depth were found for the mushroom coral *Podabacia* sp. (PERMANOVA, pseudo–F = 53.2, $p < 0.001$; Table 4.2), which exhibited significantly higher numbers of relative *nifH* gene copies at 10 and 20 m water depth (38–fold and 23–fold, respectively), compared to their conspecifics sampled at 30 m (pair–wise PERMANOVA, 10–30 m: $t = 8.61$, $p = 0.004$; 20–30 m: $t = 10.82$, $p < 0.001$; Figure 4.1A). These fold–changes correspond to a

decrease in relative *nifH* gene copy numbers of 97 % with water depth. *Pachyseris speciosa* exhibited 9–fold and 11–fold higher numbers of relative *nifH* gene copies at 30 and 40 m, respectively, compared to 50 m, but these differences were statistically not significant (PERMANOVA, pseudo–F = 1.69, p = 0.230; Figure 4.1B and Table 4.2). These fold–changes correspond to a decrease in relative *nifH* gene copy numbers of 90 % with water depth. Overall, variation of biological replicates was greater in *Pachyseris speciosa* than in *Podabacia* sp. (Figure 4.1).

Table 4.3 | Pairwise comparison of six environmental parameters between water depths (m).

A

	1	10	20	30	40	50
1	X					
10	*	X				
20	***	***	X			
30	***	***	***	X		
40	***	***	***	***	X	
50	***	***	***	***	***	X

B

	1	10	20	30	40	50
1	X					
10	***	X				
20	***	***	X			
30	***	***	***	X		
40	***	***	***	***	X	
50	***	***	***	***	***	X

C

	1	10	20	30	40	50
1	X					
10	ns	X				
20	**	*	X			
30	*	ns	ns	X		
40	***	***	***	***	X	
50	***	***	***	***	ns	X

D

	1	10	20	30	40	50
1	X					
10	**	X				
20	***	ns	X			
30	***	***	*	X		
40	***	ns	ns	***	X	
50	ns	ns	ns	***	ns	X

E

	1	10	20	30	40	50
1	X					
10	ns	X				
20	ns	ns	X			
30	ns	ns	ns	X		
40	ns	ns	ns	ns	X	
50	ns	ns	ns	ns	ns	X

F

	1	10	20	30	40	50
1	X					
10	***	X				
20	***	***	X			
30	***	***	*	X		
40	***	***	***	ns	X	
50	***	***	***	ns	ns	X

(A) Temperature, **(B)** Photosynthetic Active Radiation (PAR), **(C)** dissolved oxygen (O_2), **(D)** salinity, **(E)** turbidity, and **(F)** chlorophyll concentration. Obtained by pair–wise PERMANOVA. ns = not significant, * = significant (* < 0.05; ** < 0.01; *** < 0.001)

Environmental parameters

All environmental parameters, except for turbidity, showed significant differences with depth (PERMANOVA, p < 0.001; for full details, see Table 4.2). Temperature decreased by 3.8 °C from 10 to 50 m water depth (30.1 and 26.3 °C, respectively; Figure 4.2A) (pair–wise PERMANOVA, t = 40.21, p < 0.001). PAR exhibited an exponential decrease with water depth. Approximately 6% of the light available at 10 m water depth around noon (561 µmol photons m^{-2} s^{-1}) penetrated down to 50 m water depth (35 µmol photons m^{-2} s^{-1}) (Figure 4.2B). Both temperature and PAR decreased with each successive depth measurement (pair–wise PERMANOVA, p < 0.001). Dissolved O_2 concentration decreased from 5.48 mg L^{-1} at 10 m to 5.28 mg L^{-1} at 50 m (pair–wise PERMANOVA, t = 10.51, p < 0.001; Figure 4.2C). Salinity decreased between 10 and 30 m from 39.4 PSU to 39.3 PSU (pair–wise PERMANOVA, t = 6.01, p < 0.001; Figure 4.2D), but increased again between 30 and 50 m from 39.3 PSU to 39.4 PSU (pair–wise PERMANOVA, t = 5.12, p < 0.001; Figure 4.2D). Turbidity remained stable along the water depth gradient (Figure 4.2E). Chlorophyll concentration increased significantly from 0.14 mg m^{-3} at 10 m to 0.72 mg m^{-3} at

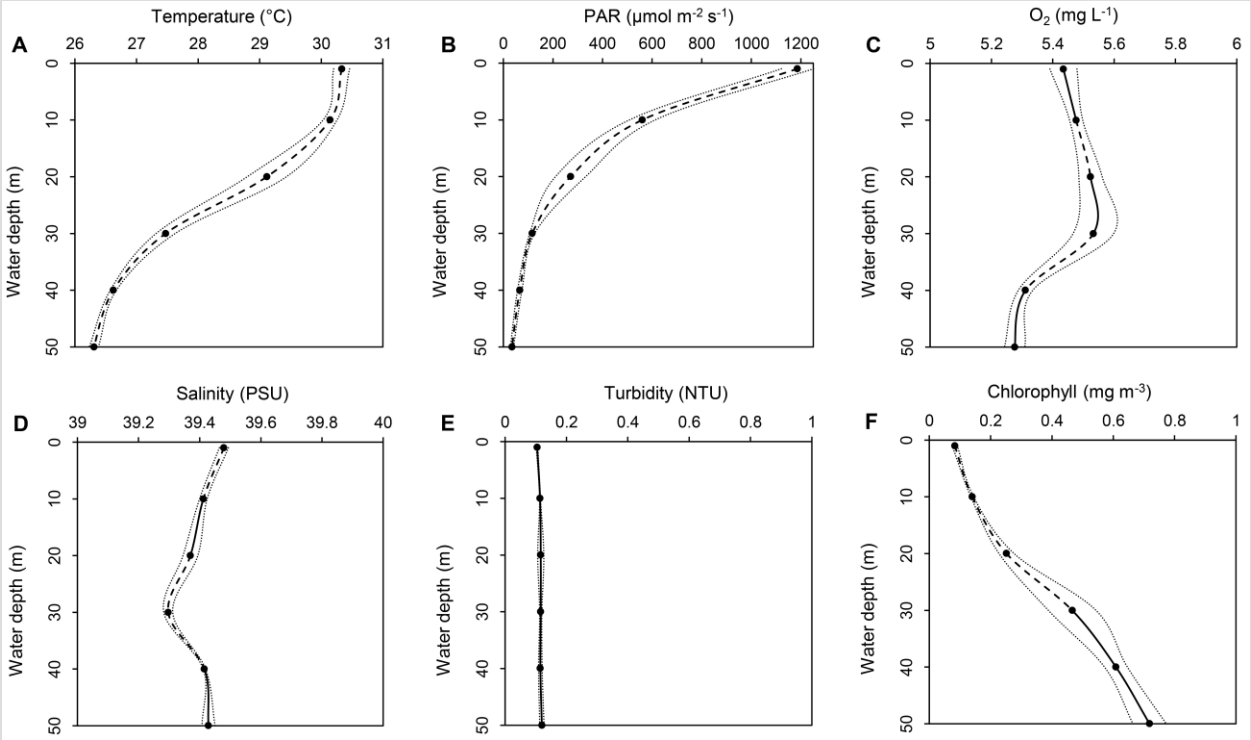

Figure 4.2 | Means of six environmental parameters, i.e., **(A)** temperature, **(B)** photosynthetic active radiation (PAR), **(C)** dissolved oxygen (O_2), **(D)** salinity, **(E)** turbidity, and **(F)** chlorophyll concentration, along a 50 m water depth gradient measured every 10 m. Data are presented as means ± 95% confidence intervals (dotted lines). Dashed lines represent significant differences between subsequent data points ($p < 0.05$). Solid lines represent non–significant differences between subsequent data points. Modified from Ziegler et al.[29].

50 m (pair–wise PERMANOVA, $t = 16.90$, $p < 0.001$; Figure 4.2F). See Table 4.3 for full details on pair–wise comparisons between all depths per environmental parameter.

In general, environmental parameters changed more drastically along the depth distribution of *Podabacia* sp. compared to those along the depth distribution of *Pachyseris speciosa*: temperature decreased with 2.7 and 0.8 °C, respectively; PAR decreased with 444 µmol m^{-2} s^{-1} and 82 µmol m^{-2} s^{-1}, respectively; and chlorophyll concentrations increased by 0.33 mg m^{-3} and 0.25 mg m^{-3}, respectively. Oxygen increased by 0.06 mg L^{-1} along the depth distribution of *Podabacia* sp., while it decreased with 0.26 mg L^{-1} along the depth distribution of *Pachyseris speciosa*.

Relationships of relative nifH *gene copy numbers with environmental parameters*

No correlation was found between depth and Symbiodiniaceae cell density for *Podabacia* sp. (Linear Regression, $F = 0.059$, $r^2 = 0.008$, $p = 0.82$; Figure 4.3A) and *Pachyseris speciosa* (Linear Regression, $F = 0.006$, $r^2 = 0.001$, $p = 0.94$; Figure 4.3B). A negative correlation was found between depth and relative *nifH* gene copy numbers (Linear Regression, $F = 12.1$, $r^2 = 0.388$, $p = 0.003$; Figure 4.4), i.e., decreasing relative *nifH* gene copy numbers with increasing depth. The environmental parameter that best explained this pattern in the BIOENV routine was temperature ($r = 0.317$, $p < 0.01$; Supplementary Figure S4.2), followed by chlorophyll concentration ($r = 0.289$), PAR ($r = 0.269$) and dissolved O_2 ($r = 0.105$).

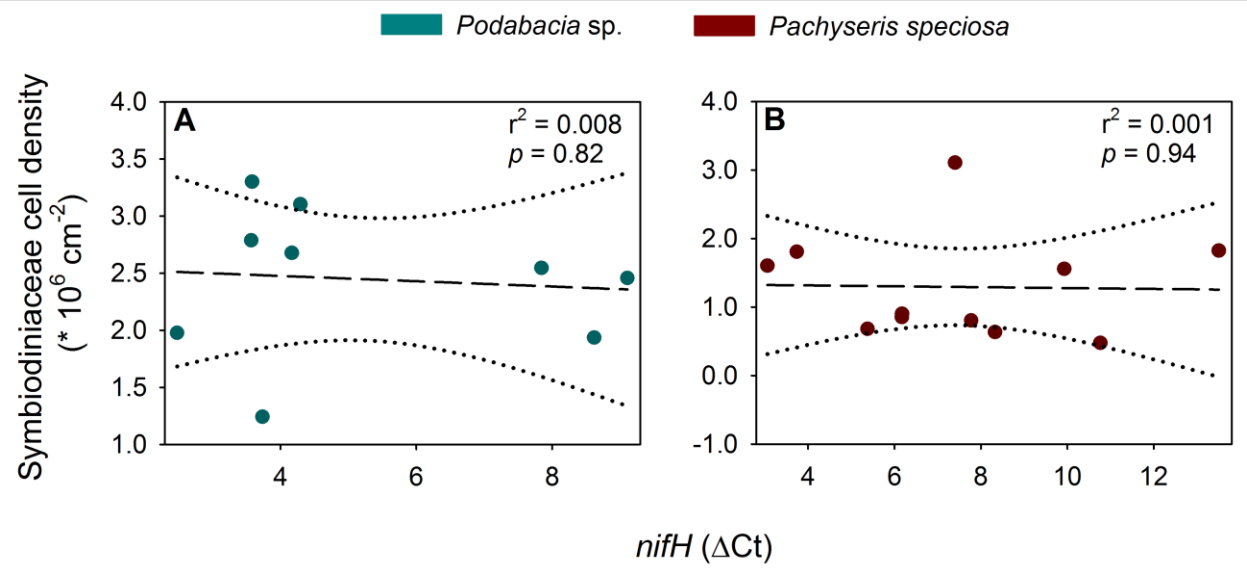

Figure 4.3 | Linear regression analyses for cell density of Symbiodiniaceae and ΔCt of *nifH* (relative to the corresponding 16S rRNA Ct value) for *Podabacia* sp. **(A)** and *Pachyseris speciosa* **(B)**. Dotted lines represent 95 % confidence intervals. Symbiodiniaceae cell density data was obtained from Ziegler et al.[29].

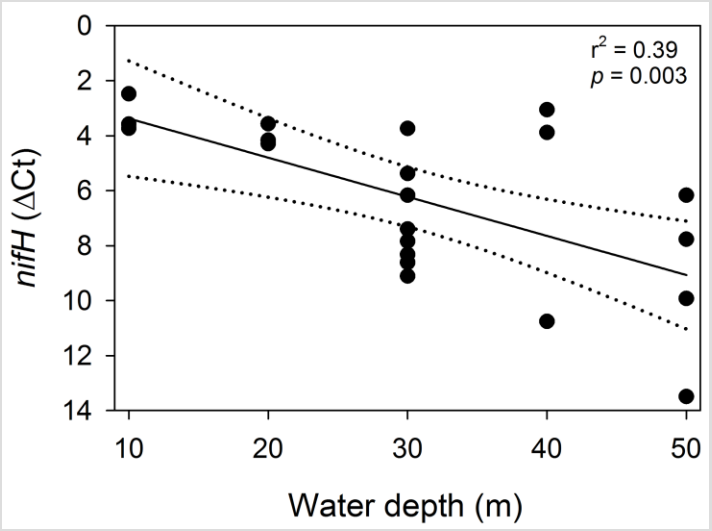

Figure 4.4 | Linear regression analysis for ΔCt of *nifH* (relative to the corresponding 16S rRNA Ct value) and water depth. Dotted lines represent 95 % confidence intervals. Note that lower ΔCt values correspond with higher relative *nifH* gene copy numbers.

4.5 | Discussion

In order to explore patterns of N_2 fixation activity along a depth gradient, we assessed relative *nifH* gene copy numbers in the tissues of two scleractinian corals species on a central Red Sea coral reef. Independent of their respective depth distribution range, which overlapped little, both coral species exhibited decreased relative *nifH* gene copy numbers at their greatest sampling depth. Temperature decreased from

30.1 °C at 10 m water depth to 26.3 °C at 50 m water depth and was, of the parameters tested, the environmental variable that best explained the observed patterns.

At the deepest sampling depths, *nifH* gene abundances for *Podabacia* sp. and *Pachyseris speciosa* were ~97 % and ~90 % lower, respectively, relative to conspecifics collected from shallower depths. This suggests a differential ecological dependence of these coral species on diazotrophy along their depth distribution range, as observed previously in other corals[17]. This may be related to a shift from autotrophic to heterotrophic metabolism of the coral holobiont with increasing water depths. Indeed, coral species with higher heterotrophic potential may harbor lower abundances of diazotrophs in their tissue[12]. Furthermore, translocation of photosynthate from Symbiodiniaceae to the coral host, potentially fueling the energy–expensive activity of coral–associated diazotrophs[13], are reported to decrease from > 90 % to around 30 % along a (mesophotic) depth gradient in the coral *Stylophora pistillata* due to changes in holobiont resource partitioning[36]. Thus, the observed decrease in relative *nifH* gene copy numbers, i.e., relative diazotroph abundances, with increasing water depth may indeed be linked to reduced photosynthate translocation by Symbiodiniaceae[8,25]. Other studies have reported increased dependence on heterotrophy by coral holobionts with increasing water depth, decreasing temperature and/or light availability[22,37–42]. Thereby, heterotrophy could provide the coral holobiont with a large part of its nutrient demand at these greater depths[27,43–45]. However, this depth–dependent rate of N_2 fixation may be less pronounced when the supply of heterotrophic resources is not limited[22,46]. It is therefore noteworthy to mention that data obtained here were collected in the highly oligotrophic Red Sea, with potentially limited heterotrophic resource abundance.

The results obtained here support the observation of low diazotroph abundances that align with increased heterotrophic potential. While previously investigated in a comparative taxonomic framework[12], we here provide a spatial context. Both temperature and PAR decreased with depth at rates that are comparable to measurements in other coral reef systems[24,47,48]. Out of the measured parameters, temperature was the environmental variable that best explained the observed patterns in the present study. Indeed, temperature is a key environmental driver of N_2 fixation activity and diazotroph abundance in scleractinian corals[13,49,50]. Notably, seawater temperature decreased more strongly over the depth distribution range of *Podabacia* sp. than over the distribution range of *Pachyseris speciosa*. Thus, more pronounced differences in environment along the vertical depth distribution of *Podabacia* sp. compared to *Pachyseris speciosa* may explain the more pronounced differences in relative *nifH* relative gene copy numbers between sampling depths and coral species. Chlorophyll concentration was the second-best environmental variable to explain the relative diazotrophic abundance pattern with depth. While no direct link between ambient chlorophyll concentrations and N_2 fixation is known, the increasing chlorophyll concentration with depth could be due to increased nutrient availability[51], potentially leading to "ammonia switch–off"[19], and/or increased abundance of zooplankton that graze on phytoplankton. Thus, potentially more N is available for heterotrophic uptake for the corals. This may, in part, explain the decreasing relative *nifH* gene copy numbers and supports the hypothesis of increased heterotrophic potential at greater depths.

Pachyseris speciosa appears to prefer a low light regime[52]. Indeed, *Pachyseris speciosa* at our study site was only found in deeper (upper mesophotic) waters. Previously, Ziegler et al.[29] demonstrated that the acclimatization potential of Red Sea corals with mesophotic distribution is related to their algal symbiont physiologies, which is first and foremost driven by PAR (and interactions of other environmental

parameters). In the present study, relative *nifH* gene copy numbers decreased with decreasing PAR despite a lack of correlation with cell densities of their photosynthetic algal symbiont. PAR positively correlates with N_2 fixation activity in benthic coral reef organisms[53,54], while increased O_2 may negatively affect the nitrogenase enzyme complex[18]. While the latter may in part explain the pattern observed in *Podabacia* sp. between 10 and 20 m water depth, the opposite effect is found at all other depths for both species. Additionally, many diazotrophs have mechanisms in place to protect them from the potentially harmful effect of O_2 on the nitrogenase enzyme[25,55]. Ambient dissolved O_2 measured may therefore not be a main driver of relative *nifH* gene copy numbers in the present study.

It is worth mentioning that the central Red Sea experiences pronounced seasonal changes in environmental parameters[56,57]. The period of study (i.e., fall) is characterized by stratification with fairly stable environmental parameters[57], which is followed in winter by mixing and thus less pronounced differences in environmental parameters with depth. Thus, the results obtained here may not be representable for this site and these species throughout the entire year but merely during this season. Furthermore, the high variability of relative *nifH* gene copy numbers in *Pachyseris speciosa* may be due to the limited number of replicates used in the present study. For future research, this could be overcome by collecting a higher number of replicates.

In summary, our study suggests that corals in shallow water may rely more heavily on N acquisition through N_2 fixation. In contrast, deep(er) water conspecifics likely rely more heavily on heterotrophy for nutrient acquisition, potentially conveying high intraspecific trophic plasticity. Results obtained here contribute to a growing body of knowledge highlighting fine–scale ecological differences of corals along their water depth distribution range[58], and underline the importance of interpreting microbial functions and associated nutrient cycling processes within the holobiont in relation to water depth.

4.6 | Acknowledgments

We thank KAUST CMOR staff and boat crews for their support with diving operations, in particular C. Walcher, D. Pallett, T. Sinclair−Taylor, T. Habis, and F. Mallon. We also thank C. Roder for assistance with coral collection and A. Baird for assistance in the taxonomic assignment of the coral species. MZ was financially supported by a DAAD postgraduate research fellowship. Financial support was provided by KAUST baseline funds to CRV and the German Research Foundation (DFG) grant Wi 2677/9−1 to CW.

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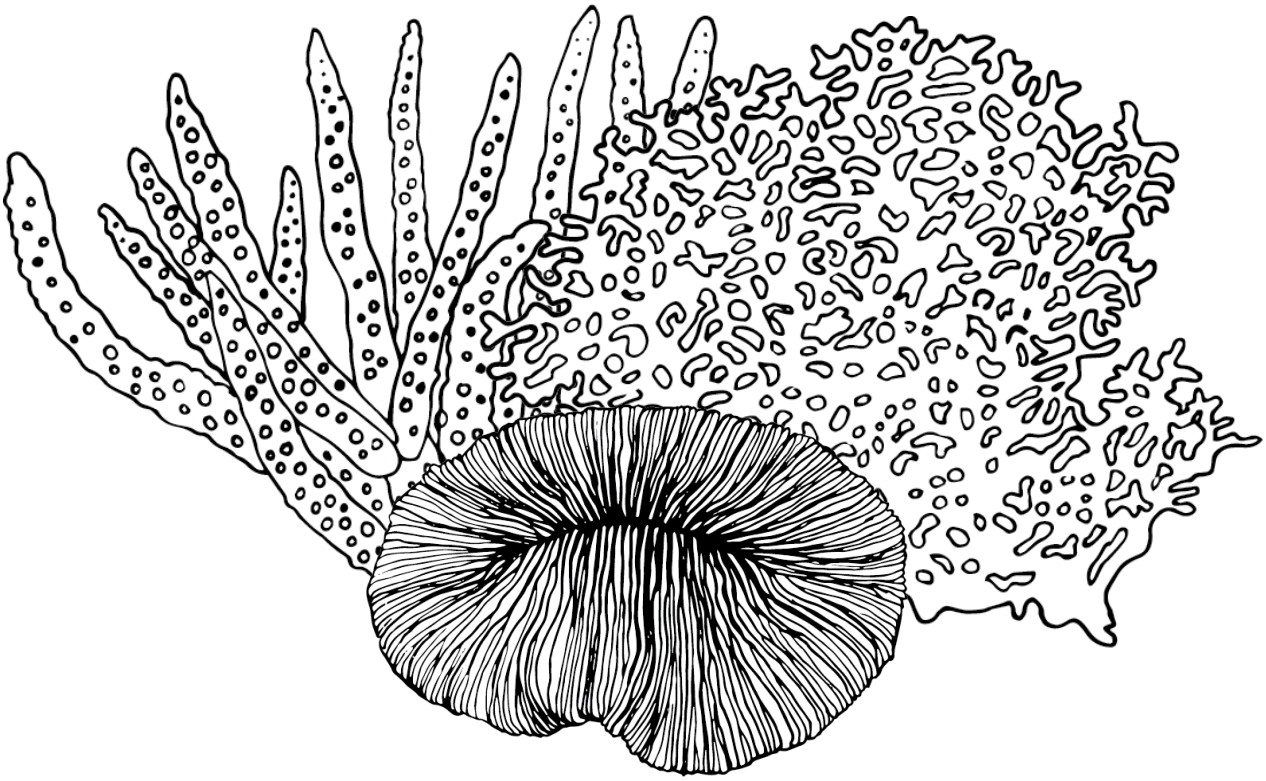
Chapter 5



Chapter 5 | **Denitrification aligns with N_2 fixation in Red Sea corals**

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5.1 | Abstract

Denitrification may potentially alleviate excess nitrogen (N) availability in coral holobionts to maintain a favourable N to phosphorous ratio in the coral tissue, however, little is known about the activity of denitrifiers. The present study used the *nirS* marker gene as a proxy for denitrification potential along with measurements of denitrification rates in a comparative taxonomic framework from the Red Sea: *Acropora hemprichii*, *Millepora dichotoma*, and *Pleuractis granulosa*. Relative *nirS* gene copy numbers associated with the tissues of these common corals were assessed and compared with denitrification rates on the holobiont level. In addition, dinitrogen (N_2) fixation rates, Symbiodiniaceae cell density, and oxygen evolution were assessed to provide an environmental context for denitrification. Findings revealed that relative abundances of the *nirS* gene were 16− and 17−fold higher in *A. hemprichii* compared to *M. dichotoma* and *P. granulosa*, respectively. In concordance, highest denitrification rates were measured in *A. hemprichii*, followed by *M. dichotoma* and *P. granulosa*. Denitrification rates were positively correlated with N_2 fixation rates and Symbiodiniaceae cell densities. Our results suggest that denitrification may counterbalance the N input from N_2 fixation in the coral holobiont, and we hypothesize that these processes may be limited by photosynthates released by the Symbiodiniaceae.

Keywords: Coral reefs | diazotrophy | nitrogen cycling | Anthozoa | Hydrozoa | Symbiodiniaceae

A modified version of this chapter has been published in *Scientific Reports* 9:19460.
https://doi.org/10.1038/s41598-019-55408-z

5.2 | Introduction

Corals are holobionts consisting of the coral host and a diverse microbiome composed of Symbiodiniaceae (i.e., endosymbiotic dinoflagellates capable of photosynthesis), and prokaryotes, i.e. bacteria and archaea, among other microbes[1]. Complex symbiotic interactions within these holobionts render corals mixotrophic, that is they can obtain nutrients through both autotrophic and heterotrophic means[2–4]. The endosymbiotic dinoflagellates, belonging to the family Symbiodiniaceae[5], provide the coral with a substantial part of their metabolic energy via autotrophy in the form of photosynthetically fixed carbon (C)[6]. In return, the Symbiodiniaceae require nutrients from the coral host, e.g. nitrogen (N) and phosphorous (P), which can be obtained via heterotrophic feeding or by uptake from the water column and/or internal (re)cycling[7,8].

The involvement of prokaryotes in holobiont nutrient cycling has received increasing attention in recent years. Diazotrophs in particular (microbes capable of fixing atmospheric dinitrogen (N_2)) ubiquitously occur in corals[9–11] and are recognized as an important source of N for holobiont productivity[12–16]. Diazotrophs can provide the holobiont with bioavailable N in the form of ammonium, a preferred N source for Symbiodiniaceae[17–19], in particular in times of N scarcity[11].

Excess (microbial) input of bioavailable N into the coral holobiont can potentially lead to a misbalance of the N:P ratio, i.e. a shift from N towards P limitation, thereby increasing bleaching susceptibility[14,20,21]. Previously, it was hypothesized that the activity of other N−cycling microbes could alleviate coral holobionts from nutrient stress via the removal of nitrogenous compounds[22]. Indeed, ammonium oxidizing (i.e. nitrifying) and nitrate reducing (i.e. denitrifying) prokaryotes occur ubiquitously on coral reefs[23–27], including coral holobionts[22,28,29]. The denitrification pathway in particular may be important for holobiont functioning as it effectively removes bioavailable N. Here, nitrate is reduced to atmospheric N_2 via the activity of four main enzymes, i.e. nitrate reductase (converting nitrate to nitrite), nitrite reductase (converting nitrite to nitric oxide), nitric oxide reductase (converting nitric oxide to nitrous oxide), and nitrous oxide reductase (converting nitrous oxide to N_2) (Figure 5.1)[30,31]. While Symbiodiniaceae cells are considered a major N sink in the coral holobiont[32], excess N could potentially be removed by denitrifying microbes to help maintain an N−limited state[14]. However, whether removal of excess N via denitrification contributes to holobiont functioning and health remains poorly understood.

Recently, Pogoreutz *et al*.[10] demonstrated that N_2 fixation rates may not only be species−specific, but align with relative gene copy numbers and expression of the *nifH* gene. This pattern was linked to heterotrophic capacity of the investigated corals. However, it is unknown how these patterns of N_2 fixation activity ultimately relate to other N−cycling processes, i.e. denitrification, within the coral holobiont. The present study thus aimed to answer (*i*) whether patterns of denitrification are coral species−specific; (*ii*) whether relative abundances of the *nirS* gene (denitrification potential) can be related to denitrification rates; and (*iii*) whether denitrification aligns with other biological variables within the coral holobiont, specifically N_2 fixation, photosynthesis, and cell density of Symbiodiniaceae. These questions were answered in a comparative taxonomic framework of three common Red Sea coral species. Relative gene copy numbers of the *nirS* gene, which encodes for a nitrite reductase containing cytochrome cd_1, were assessed by qPCR to serve as a proxy for denitrification potential of coral tissue−associated prokaryotes. Relative quantification of *nirS* gene copy numbers was achieved by referencing against the ITS2 region of Symbiodiniaceae. Denitrification and N_2 fixation rates were quantified indirectly using a COmbined

Blockage/Reduction Acetylene (COBRA) assay[33]. Finally, Symbiodiniaceae cell densities were manually counted and photosynthesis was assessed by measuring O_2 fluxes.

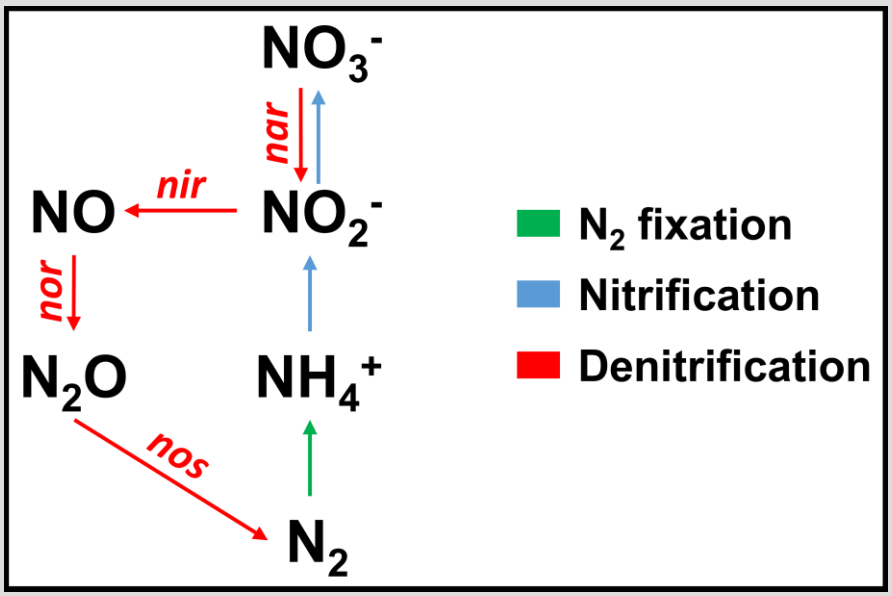

Figure 5.1 | Schematic representation of three major pathways involved in nitrogen cycling, including the four gene clusters responsible for denitrification. N_2 = atmospheric nitrogen, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, NO = nitric oxide, N_2O = nitrous oxide, *nar* = gene cluster for nitrate reductase, *nir* = gene cluster for nitrite reductase, *nor* = gene cluster for nitric oxide reductase, *nos* = gene cluster for nitrous oxide reductase.

5.3 | Materials and Methods

Sample collection, aquarium facilities, and maintenance

This study was conducted at the King Abdullah University of Science and Technology (KAUST) in Saudi Arabia. Three common coral species were selected (Figure 5.2A) and collected at approx. 5 m water depth at the semi−exposed side of the inshore reef Abu Shoosha (N22°18'15", E39°02'56") located in the Saudi Arabian central Red Sea in November 2017; specifically, the acroporid coral *Acropora hemprichii* (*n* = 4 colonies), the hydrozoan *Millepora dichotoma* (*n* = 4 colonies), and the fungiid coral *Pleuractis granulosa* (*n* = 8 polyps). Coral colonies of the same species were sampled at least 10 m apart to account for genetic diversity. After collection, the corals were transferred to recirculation aquaria filled with reef water on the vessel, and subsequently transported to the wet lab facility of the Coastal and Marine Resources (CMOR) Core Lab at KAUST. The branching corals *A. hemprichii* and *M. dichotoma* were immediately fragmented into two fragments of similar size each. Fragments were distributed into four independent replicate 150 L flow−through tanks, i.e. each tank held two distinct fragments of each branching coral species (see Supplementary Figure S5.1). Individual polyps of *P. granulosa* were not fragmented and distributed randomly over the four tanks. Fragments/polyps were left to acclimate for two weeks prior to the start of measurements. All tanks were continuously supplied with sediment−filtered seawater (flow through rate 300 L h^{-1}) from inshore reefs located 1.5 km off KAUST with the following parameters: temperature 27 °C, salinity 40 PSU, and dissolved oxygen (O_2) 6.4 mg O_2 L^{-1}. All fragments were exposed to a photon flux of ~150 µmol m^{-2} s^{-1} [34] on a 12:12 h light/dark cycle. Corals were

kept in nutrient−rich seawater (nitrate ~3 µM, phosphate ~0.40 µM) to stimulate denitrification response in coral holobionts[35–37]. For each measurement, one fragment/polyp of each species per tank was taken, avoiding sampling fragments that originated from the same colony. This resulted in four fragments/polyps per species from four different colonies for each measurement (see Supplementary Figure S5.1).

DNA extraction and relative quantification of the nirS *gene via quantitative PCR (qPCR)*

Quantitative PCR was used to quantify relative gene copy numbers of the *nirS* gene as a proxy for abundance of denitrifying prokaryotes in the coral tissues, i.e. denitrification potential. To this end, the coral tissue was removed from the skeleton by airblasting with RNase free water and pressurized air using a sterilized airbrush (Agora−Tec GmbH, Schmalkalden, Germany). For *P. granulosa*, tissue was blasted off from both top and bottom surfaces and was pooled subsequently. The resulting tissue slurries were homogenized using an Ultra Turrax (for approx. 20 s) and stored at −20 °C until further processing. Total DNA was extracted from 100 µL of tissue slurry using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer's instructions. DNA extraction yields were determined using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentrations were adjusted to 2 ng μL^{-1} and stored at −20 °C until further processing.

qPCR assays were performed in technical triplicates for each coral fragment or polyp. Each assay contained 9 µL reaction mixture and 1 µL DNA template. Reaction mixture contained Platinum SYBR Green qPCR Master Mix (Invitrogen, Carlsbad, CA, United States), 0.2 µL of each primer (10 µM, see below and details of primer assessment in Chapter 6), 0.2 µL of ROX dye and 3.4 µL of RNAse−free water. Negative controls (i.e., reactions consisting of only qPCR reagents and nuclease−free water without any DNA added) were included in the assay in technical triplicates to account for potential laboratory and kit contamination. The relative number of *nirS* gene copies (i.e. relative abundance of denitrifiers) was determined by normalization against the multi copy gene marker ITS2 of Symbiodiniaceae as previously used for the normalization of *nifH* gene copy numbers in a comparative taxonomic coral framework[10]. A total of 18 primers covering all main enzymes in the denitrification pathway (Figure 5.1) were tested for this study and yielded ten primer pairs that produced PCR products in the suggested size range (see details of primer assessment in Chapter 6). Temperature gradient PCRs were applied (from 51 °C to 62 °C) to assess the optimal annealing temperature of every primer pair (see details of primer assessment in Chapter 6). Due to substantial differences in amplification performance of primer pairs, we selected a primer pair for *nirS* which encodes for a nitrite reductase containing cytochrome cd_1 as the target gene. For the amplification of *nirS*, the primers cd3aF 5'−GTSAACGTSAAGGARACSGG−3' and R3cd 5'−GASTTCGGRTGSGTCTTGA−3' were used[38]. This primer pair was previously found to perform well with DNA from other marine templates, such as coral rock[24], marine sediments[39], as well as environmental samples from intertidal zones[40], and terrestrial ecosystems[41–43]. To amplify the ITS2 region of Symbiodiniaceae the primers SYM_VAR_5.8S2 5'−GAATTGCAGAACTCCGTGAACC−3' and SYM_VAR_REV 5'−CGGGTTCWCTTGTYTGACTTCATGC−3' were used[44]. The thermal cycling protocol used for the amplification of both target genes was 50 °C for 2 min, 95 °C for 2 min, 50 cycles of 95 °C for 30 s, 51 °C for 1 min, 72 °C for 1 min, and a final 72 °C extension cycle for 2 min. Amplification specificity was determined by adding a dissociation step. All assays were performed on the ABI 7900HT Fast Real−Time PCR System (Applied Biosystems, CA, USA). Standard calibration curves were run simultaneously covering 5 orders of magnitude (10^3–10^7 copies of template per assay for the ITS2 and *nirS* gene). The qPCR efficiency (E) of

both primer pairs was 84 % and 86 %, respectively, calculated according to the equation $E = [10^{(-1/slope)}-1]$. Relative fold change of *nirS* gene copies were calculated as $2^{(-\Delta\Delta Ct)}$ against ITS2 Ct values using *P. granulosa* samples as the reference.

Throbäck *et al*.[45] assessed a range of *nirS* primer pairs and concluded that the primer pair used in the present study (i.e. cd3aF and R3cd) had the largest range and worked best for *nirS* gene assessments. Currently, there are no optimal universal primers for the amplification of the *nirS* gene available[46]. Any quantification of *nirS* abundances is hence biased by the primer pair used and its suitability strongly depends on the phylogenetic diversity of the template. Thus, while the primer combination used here shows a high coverage of 67 % of known *nirS* diversity[46], our results can only provide an approximation of the relative abundance of denitrifying bacteria across samples until more (meta)genomic data for coral−associated denitrifiers are available.

Denitrification and N_2 fixation measurements

To measure denitrification and N_2 fixation rates simultaneously, we incubated corals using a COBRA assay, as described in El−Khaled et al.[33]. Of note, acetylene inhibits the production of nitrate in the nitrification pathway[47,48]. As nitrate serves as a substrate for denitrification, the inhibition of nitrification may thus result in an underestimation of denitrification rates. To compensate for such effects, nutrient−rich incubation water was used to preclude substrate limitation[35–37].

Briefly, incubations were conducted in gas−tight 1 L glass chambers, each filled with 800 mL of nutrient−rich sediment−filtered seawater (DIN = ∼3 µM, phosphate = ∼0.40 µM) and a 200 mL gas headspace. Both incubation water and headspace were enriched with 10% acetylene. Each chamber contained a single *A. hemprichii* or *M. dichotoma* fragment or *P. granulosa* polyp. Incubations of four biological replicates per species were performed (see Supplementary Figure S5.1), and three additional chambers without corals served as controls to correct for planktonic background metabolism. During the 24 h incubations, chambers were submersed in a temperature−controlled water bath and constantly stirred (500 rpm) to create a constant water motion and homogenous environment (27 °C, 12:12 h dark/light cycle, photon flux of ∼150 µmol m^{-2} s^{-1}). Nitrous oxide (N_2O; as a proxy for denitrification) and ethylene (C_2H_4; as a proxy for N_2 fixation) concentrations were quantified by gas chromatography and helium pulsed discharge detection (Agilent 7890B GC system with HP−Plot/Q column, lower detection limit for both target gases was 0.3 ppm). To facilitate comparisons of both N−cycling processes, N_2O and C_2H_4 production rates were converted into N production using molar ratios of N_2O:N_2 = 1 and C_2H_4:N_2 = 3[49], and multiplying by 2 to convert N_2 to N, resulting in rates of nmol N cm^{-2} d^{-1}. Gas concentrations were normalized to coral surface area, which was calculated using cloud−based 3D models of samples (Autodesk Remake v19.1.1.2)[50,51].

Symbiodiniaceae cell density

Tissue slurry for DNA extraction was also used to obtain cell densities of Symbiodiniaceae (see Supplementary Figure S5.1). Symbiodiniaceae cell densities were obtained by manual counts of homogenized aliquots of 20 µL, which were diluted 5 times, using a Neubauer−improved hemocytometer on a light microscope with HD camera (Zeiss, Germany). Resulting photographs were analysed using the Cell Counter Notice in ImageJ software (National Institutes of Health, USA). Cell counts for each individual

were done in duplicates and subsequently averaged. Finally, cell counts were normalized to coral surface area to obtain cell densities of Symbiodiniaceae for each fragment or polyp.

O_2 fluxes

Net photosynthesis (P_n) and dark respiration (R) were assessed from O_2 evolution/depletion measurements with the same fragments/polyps 2 days prior to using them for denitrification and N_2 fixation rate measurements. Corals were incubated for 2 h in individual gas−tight 1 L glass chambers, filled with nutrient−rich sediment−filtered seawater (DIN = ∼3 µM, phosphate = ∼0.40 µM). Each chamber contained a single *A. hemprichii* or *M. dichotoma* fragment or *P. granulosa* polyp. Incubations of four biological replicates per species were performed (see Supplementary Figure S5.1), and three additional chambers without corals served as controls to correct for planktonic background metabolism. During the incubations, chambers were submersed in a temperature−controlled water bath (kept at 27 °C) and constantly stirred (500 rpm) to create a continuous water motion and homogenous environment. Light incubations for P_n were performed under a photon flux of ∼150 µmol m^{-2} s^{-1}. R was obtained by incubating in complete darkness. O_2 concentrations were measured at the start and end of the respective incubation period using an optical oxygen multiprobe (WTW, Germany). O_2 concentrations at the start of the incubation were subtracted from O_2 concentrations at the end, corrected for controls and normalized to incubation time and surface area of the corals. R is presented as a negative rate. Finally, gross photosynthesis (P_g) was calculated as the difference between P_n and R as follows: $P_g = (P_n) - (R)$

Statistical analyses

Data were analysed using non−parametric permutational multivariate analysis of variance (PERMANOVA) using PRIMER−E version 6 software[52] with the PERMANOVA+ add on[53]. To test for differences in relative *nirS* gene copy numbers, denitrification rates, N_2 fixation rates, Symbiodiniaceae cell densities and O_2 fluxes between species, 1−factorial PERMANOVAs were performed, based on Bray−Curtis similarities of square−root transformed data. Therefore, Type III (partial) sum of squares was used with unrestricted permutation of raw data (999 permutations), and PERMANOVA pairwise tests with parallel Monte Carlo tests were carried out when significant differences were found.

Differences between denitrification and N_2 fixation rates within each coral species were assessed using SigmaPlot 12.0 (Systat software). T−tests were performed for normally distributed data and Mann−Whitney U tests were performed when data were not normally distributed.

Additionally, to identify the biological variable (single trial variable) and combination of biological variables (multiple trial variables) that “best explains” the denitrification rate pattern of the coral samples, a Biota and/or Environment matching routine (BIOENV) was performed with 999 permutations based on Spearman Rank correlations. A distance−based linear model (DistLM) using a step−wise selection procedure with AICc as a selection criterion was used to calculate the explanatory power of correlating biological variables[52,54]. Finally, the same BIOENV and DistLM routine was performed for N_2 fixation rates of the coral samples.

As *P. granulosa* consisted of four individual polyps per measurement (no technical replicates originating from the same polyp), data for each variable were averaged and used as a single data point in analyses unless the same individuals were used (see Supplementary Figure S5.1). All values are given as mean ± SE.

5.4 | Results

Relative abundances of the nirS *gene and denitrification rates*

The qPCR confirmed the presence of the *nirS* gene in the tissues of all investigated corals (Figure 5.2A and Figure 5.2B). *Acropora hemprichii* exhibited significantly higher relative *nirS* gene copy numbers compared to *M. dichotoma* (~16–fold; pair–wise PERMANOVA, t = 3.82, $p = 0.015$) and *P. granulosa* (~17–fold; pair–wise PERMANOVA, t = 3.25, $p = 0.029$). A similar pattern was found for denitrification rates (Figure 5.2C). *Acropora hemprichii* exhibited the highest denitrification rates (~0.38 ± 0.13 nmol N cm^{-2} d^{-1}), followed by *M. dichotoma* (~0.17 ± 0.10 nmol N cm^{-2} d^{-1}) and *P. granulosa* (~0.05 ± 0.02 nmol N cm^{-2} d^{-1}). Denitrification rates in *A. hemprichii* were significantly different from those in *P. granulosa* (pair–wise PERMANOVA, t = 2.75, $p = 0.036$), but not those measured in *M. dichotoma* (pair–wise PERMANOVA, t = 1.31, $p = 0.237$). Finally, denitrification rates in *M. dichotoma* were not significantly different from those in *P. granulosa* (pair–wise PERMANOVA, t = 1.23, $p = 0.264$).

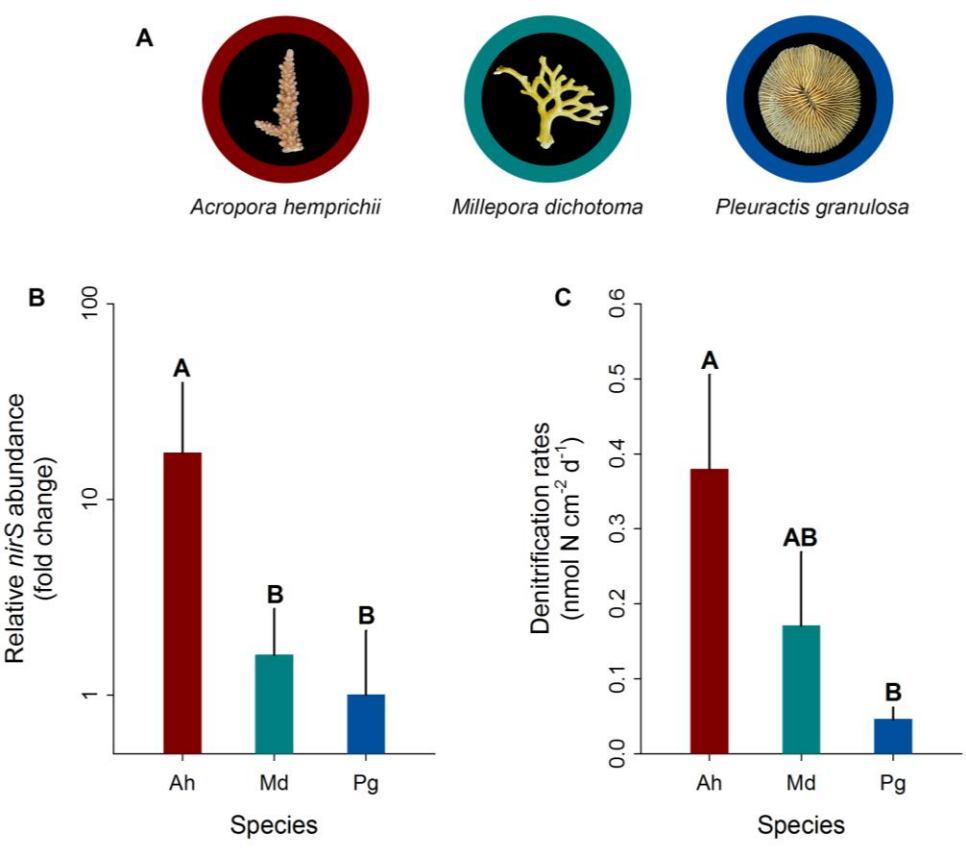

Figure 5.2 | Relative *nirS* gene copy numbers and rates of denitrification associated with three Red Sea coral species. **(A)** Representative photographs of investigated species, **(B)** fold changes in relative *nirS* gene copy numbers normalized to ITS2 copy numbers as measured by quantitative PCR, and **(C)** denitrification rates measured indirectly via the combined blockage/reduction acetylene assay (COBRA–assay). Ah = *A. hemprichii*, Md = *M. dichotoma*, and Pg = *P. granulosa*. Fold changes were calculated in relation to *P. granulosa*; bars indicate the mean; error bars indicate upper confidence intervals (+ 1 SE); $n = 4$ per species, except Ah and Pg in **(B)** ($n = 3$). Different letters above error bars indicate statistically significant differences between groups within each figure (pair–wise PERMANOVAs, $p < 0.05$).

N_2 fixation, Symbiodiniaceae cell density and O_2 fluxes

N_2 fixation rates were highest in *M. dichotoma* (0.23 ± 0.11 nmol N cm^{-2} d^{-1}), followed by *A. hemprichii* (0.21 ± 0.12 nmol N cm^{-2} d^{-1}), and lowest in *P. granulosa* (0.04 ± 0.03 nmol N cm^{-2} d^{-1}) (Figure 5.3A). Due to high biological variation in the samples, these differences between coral species were not significant (Figure 5.3A).

Cell densities of Symbiodiniaceae were significantly higher in *A. hemprichii* (0.86 ± 0.15 × 10^6 cells cm^{-2}) compared to *M. dichotoma* (0.10 ± 0.02 × 10^6 cells cm^{-2}; pair−wise PERMANOVA, t = 6.86, $p < 0.001$) and *P. granulosa* (0.13 ± 0.03 × 10^6 cells cm^{-2}; pair−wise PERMANOVA, t = 5.03, $p = 0.003$) (Figure 5.3B). Cell densities of Symbiodiniaceae in *M. dichotoma* and *P. granulosa* did not differ significantly (Figure 5.3B).

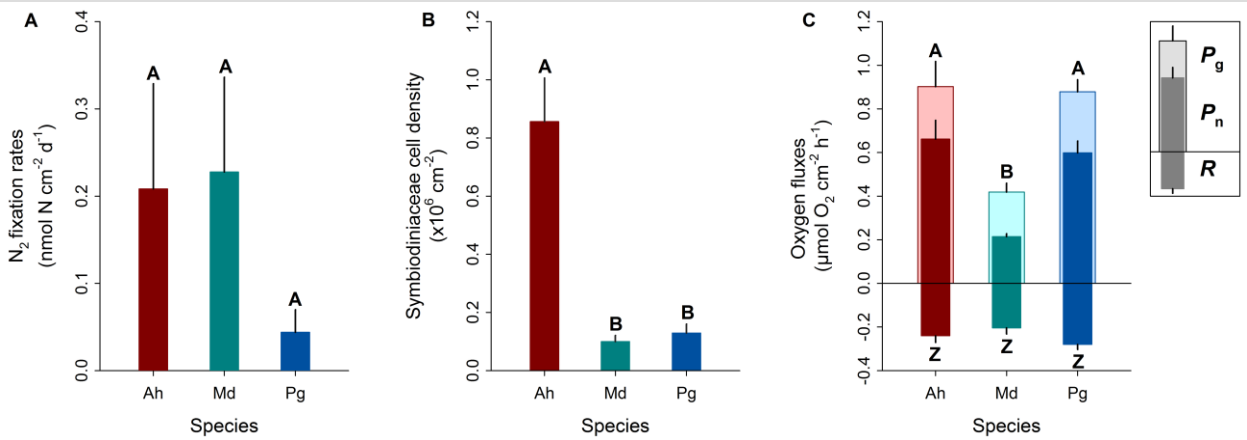

Figure 5.3 | Biological variables of three Red Sea corals. **(A)** N_2 fixation rates measured indirectly using a COBRA assay, **(B)** Symbiodiniaceae cell densities, and **(C)** oxygen fluxes. Ah = *Acropora hemprichii*, Md = *Millepora dichotoma*, and Pg = *Pleuractis granulosa*. P_g = gross photosynthesis, P_n = net photosynthesis, R = dark respiration. Bars indicate the mean; error bars indicate upper confidence intervals (+1 SE); $n = 4$ per species. Different letters above error bars indicate statistically significant differences within each plot (pair−wise PERMANOVAs, $p < 0.05$); differences in (**C**) apply to both P_g and P_n.

Significantly lower P_n was found for *M. dichotoma* (0.21 ± 0.01 µmol O_2 cm^{-2} h^{-1}) compared to that of *A. hemprichii* (0.66 ± 0.09 µmol O_2 cm^{-2} h^{-1}; pair−wise PERMANOVA, t = 6.55, $p = 0.002$) and that of *P. granulosa* (0.60 ± 0.05 µmol O_2 cm^{-2} h^{-1}; pair−wise = PERMANOVA, t = 8.95, $p < 0.001$) (Figure 5.3C). The same pattern was found for P_g; *M. dichotoma* exhibited significantly lower P_g (0.42 ± 0.04 µmol O_2 cm^{-2} h^{-1}) than that of *A. hemprichii* (0.90 ± 0.12 µmol O_2 cm^{-2} h^{-1}; pair−wise PERMANOVA, t = 4.14, $p = 0.007$) and that of *P. granulosa* (0.88 ± 0.06 µmol O_2 cm^{-2} h^{-1}; pair−wise PERMANOVA, t = 6.21, $p = 0.002$) (Figure 5.3C). No significant differences were found for R between species (PERMANOVA, pseudo−F = 1.73, $p = 0.243$) (Figure 5.3C).

Comparison of denitrification rates and N_2 fixation rates

No significant differences were found between denitrification and N_2 fixation rates for either species (T−test, $p > 0.05$; Figure 5.4).

Correlation analyses

The biological variable that best explained the denitrification rates was N_2 fixation (BIOENV, $r = 0.654$, $p = 0.004$). The combination of biological variables that explained denitrification rates best were N_2 fixation and Symbiodiniaceae cell density (BIOENV, $r = 0.592$, $p = 0.002$). Indeed, 85.6% of the variation in denitrification rates could be explained by N_2 fixation and Symbiodiniaceae cell density (DistLM).

The biological variable that best explained the N_2 fixation rates was denitrification (BIOENV, r = 0.651, p = 0.006). The combination of biological variables that explained N_2 fixation best were denitrification and Symbiodiniaceae cell density (BIOENV, $r = 0.342$, $p = 0.046$). Indeed, 82.9% of the variation in N_2 fixation rates could be explained by denitrification and Symbiodiniaceae cell density (DistLM).

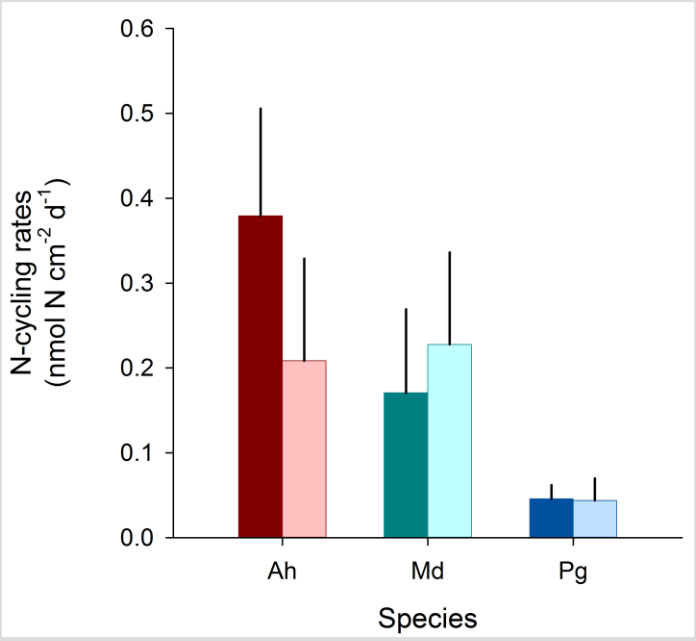

Figure 5.4 | Comparison of denitrification rates and N_2 fixation rates of three Red Sea coral species. Ah = *Acropora hemprichii*, Md = *Millepora dichotoma*, and Pg = *Pleuractis granulosa*. Bars indicate the mean; error bars indicate upper confidence intervals (+1 SE); dark bars represent denitrification rates; light bars represent N_2 fixation rates; $n = 4$ per species.

5.5 | Discussion

Despite the importance of N as a key nutrient for the metabolism of symbiotic corals[55], relatively little is known about the removal of N by microbes in the internal N−cycling of coral holobionts[12,14]. Here, we assessed relative abundances of coral tissue−associated denitrifiers (using relative gene copy numbers of the *nirS* gene as a proxy), as well as denitrification and N_2 fixation rates on the holobiont level in a comparative taxonomic framework using three common Red Sea corals. Our results suggest that denitrification is an active N−cycling pathway in coral holobionts and may be linked with diazotroph activity and Symbiodiniaceae cell density, the interplay of which may have important implications for coral holobiont nutrient cycling.

It was previously hypothesized that coral associated N−cycling microbes may have a capacity to alleviate nutrient stress via the removal of bioavailable N[14,22,28,29]. While the community structure and phylogenetic diversity of denitrifying microbes have been previously assessed in a soft and a hard coral[29], we here present the first study to link relative coral tissue−associated abundances of denitrifying prokaryotes with denitrification rates. Our findings highlight that denitrification may play a role in removing bioavailable N from the coral holobiont. The contribution and hence potential functional importance of denitrification may depend on the host species.

In the present study, the highest relative *nirS* gene copy numbers were found in *A. hemprichii*, while lower relative numbers were observed in *M. dichotoma* and *P. granulosa*. The patterns in relative abundance of the *nirS* gene obtained through qPCR were largely reflected in denitrification rates measured using a COBRA assay. As such, these data suggest that relative *nirS* gene abundance may be a suitable proxy of denitrification potential in corals. Small deviations in the patterns observed for both measurements may be potentially explained by a) differences in the community composition of denitrifying microbes; fungi involved in N metabolism may be present in coral holobionts[56] and likely lack a (homologous) *nirS* gene[57], by b) the multi−copy nature of the ITS2[58,59]; by relating *nirS* to ITS2, the relative abundances of *nirS* genes could potentially be underestimated, and by c) the potential presence of denitrifying microbes in the coral skeleton[29,60–62].

The present study identified a positive correlation between denitrification and N_2 fixation activity across the three Red Sea coral species investigated. As such, denitrification may have the capacity to counterbalance N input from N_2 fixation in coral holobionts. We here propose that these processes may be indirectly linked by their similar environmental requirements and constraints.

Denitrification as well as N_2 fixation are anaerobic processes[31,63]. However, in the present study, no relationship was found between O_2 fluxes and denitrification and N_2 fixation rates. This strongly suggests that the activity of these anaerobic processes may be spatially or temporally separated from O_2 evolution in the coral holobiont, or that the involved N−cycling prokaryotes are capable of supporting these processes in the presence of O_2[64–67]. In addition to anaerobic conditions, most denitrifiers and diazotrophs require organic C as their energy source, i.e. are heterotrophic[9,43,68,69]. Besides the uptake of organic C from the water column[70] and heterotrophic feeding by the coral host, the Symbiodiniaceae are the main source of C−rich photosynthates within the holobiont[71]. Notably, the present study showed a positive correlation between denitrification rates with N_2 fixation combined with Symbiodiniaceae cell density. In addition, a positive correlation was also shown for N_2 fixation, namely with denitrification combined with Symbiodiniaceae cell density. This suggest that the heterotrophic prokaryotes of both N−cycling pathways may rely partially on Symbiodiniaceae for obtaining organic C for respiration. As such, the correlation of denitrification and N_2 fixation may be the result of a shared organic C limitation within the holobiont[14]. However, a potential functional relationship between N−cycling prokaryotes and phototrophic Symbiodiniaceae remains yet to be determined.

The observed positive correlation between the two N−cycling pathways, i.e. denitrification and N_2 fixation, may have important implications for the general understanding of nutrient cycling within coral holobionts, and hence our understanding of coral ecology. In a stable healthy holobiont, N input from N_2 fixation may be compensated for by N removal via denitrification. As such, the activity of these two processes should be interpreted in relation to each other to understand their overall effect on holobiont N availability, and hence nutrient dynamics.

Environmental stress may directly affect the equilibrium of these processes, as both eutrophication and the increase in sea surface temperatures directly affect N−cycling within the coral holobiont[14]. Increases in inorganic N availability may lead to a reduction of diazotroph activity in coral holobionts due to the so called "ammonia switch−off"[72], which is evidenced by negative correlations between N availability and N_2 fixation for both planktonic and benthic diazotrophs[73–75]. Denitrification, on the one hand, may even be stimulated by increased nitrate availability[31]. This hypothesized interplay of denitrification and N_2 fixation would hence allow coral holobionts to effectively remove excessive N[14].

Increased sea surface temperatures, on the other hand, may directly stimulate N_2 fixation[76]. While the environmental drivers for stimulated N_2 fixation activity are not fully resolved yet, increased diazotrophy may affect holobiont functioning if not compensated for by denitrification activity[10]. However, with increasing water temperature, Symbiodiniaceae may retain more photosynthates for their own metabolism[77], potentially limiting organic C availability not only for the coral host, but also for heterotrophic microbes, including denitrifiers. Thus, microbial N−cycling may be more important in highly autotrophic coral holobionts, as they rely more on the Symbiodiniaceae for organic C and may be more susceptible to potential nutrient imbalances due to e.g. increased diazotrophic activity[10]. Indeed, the capacity for heterotrophic feeding has been linked to having a lower susceptibility to warming[78–81] and eutrophication[82]. However, besides the potential ability to remove bioavailable N from the coral holobiont, the role of denitrifiers under (non−)stressful scenarios remains speculative at this point. Thus, future research could focus on several aspects to disentangle a potential role of denitrification in the context of microbial N−cycling within coral holobionts by (a) identifying the spatial niche that denitrifiers occupy and in which abundances; (b) identifying the denitrifiers' primary energy source(s) under regular and stressed (e.g. eutrophic or warming) conditions; (c) by quantifying and assessing the interplay of denitrification with other N−cycling processes (potentially) ubiquitous in coral holobionts, e.g. N_2 fixation, nitrification and ANAMMOX, through molecular, physiological and/or isotope analyses; and (d) how the interplay of N−cycling processes in the coral holobiont is altered in global change scenarios.

5.6 | Acknowledgments

We thank KAUST CMOR staff and boat crews for their support with diving operations. Financial support was provided by KAUST baseline funds to CRV and the German Research Foundation (DFG) grant Wi 2677/9−1 to CW.

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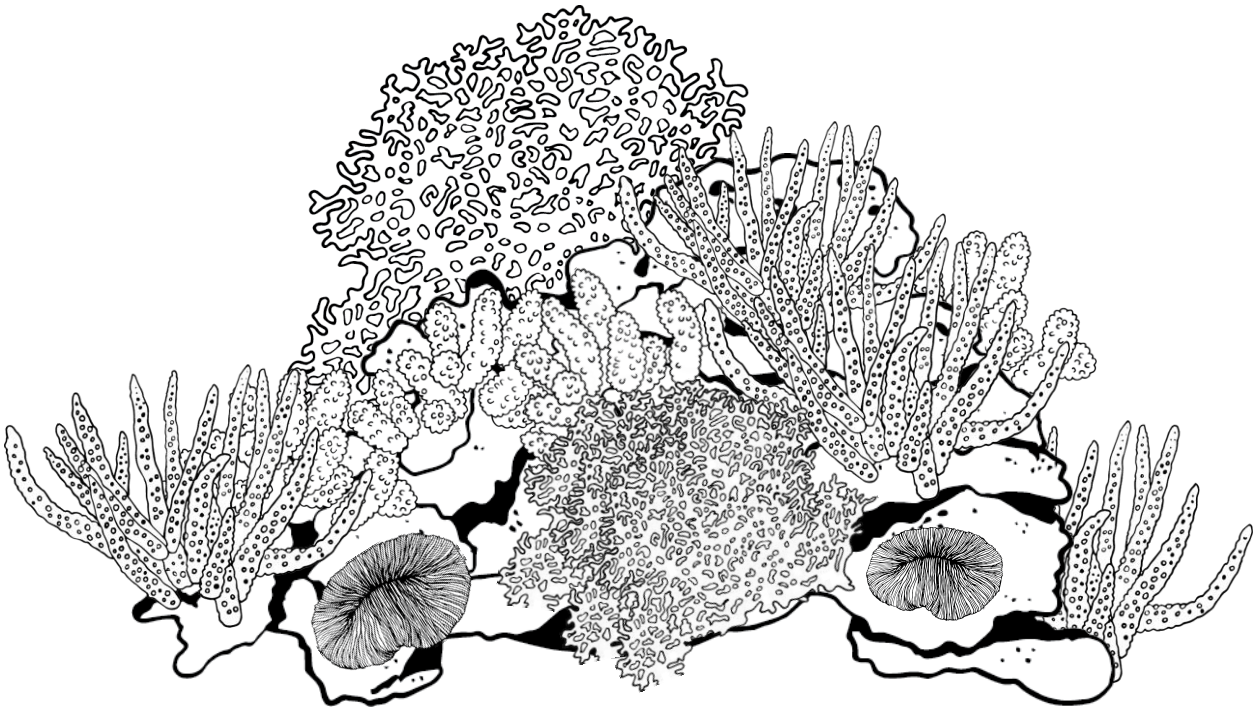
Chapter 6



Chapter 6 | **Denitrification primer assessment**

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A modified version of this chapter was published as a supplement to Chapter 5

6.1 | Approach

For the purpose of finding suitable primer pairs for the relative quantification of denitrifying prokaryotes, the selected primers were first tested with total DNA from corals previously collected for a comparative assessment of *nifH* gene copy numbers[1] in a temperature gradient PCR (range 51 to 62 °C). If one or more primers gave a single or dominant amplicon of the correct size at a given annealing temperature, the primer(s) and the annealing temperature were selected for testing quantification using quantitative PCR (qPCR). When amplification products of the same samples were similar between conventional PCR and qPCR, the primer(s) and corresponding annealing temperature were further tested on five additional common species of central Red Sea corals. Finally, all additional coral species that revealed a single and/or dominant band of the correct size were tested with qPCR.

6.2 | Material and Methods

Sample collection

This study was conducted at the King Abdullah University of Science and Technology (KAUST) in Saudi Arabia. Two species of Fungiidae (*Pleuractis granulosa* and *Ctenactis echinata*) and two species of Pocilloporidae (*Pocillopora verrucosa* and *Stylophora pistillata*) were collected (*n* = 3 colonies each) at the inshore reef Inner Fsar (22°13'97.4"N; 39°01'76.0"E) located in the Saudi Arabian central Red Sea in February 2016[1]. Five additional coral species (*n* = 3 colonies each) were collected at the inshore reef Abu Shoosha (22°18'16.3"N; 39°02'57.7"E) located in the Saudi Arabian central Red Sea in September 2017; specifically, two species of Acroporidae (*Acropora hemprichii* and *Acropora pharaonis*), one species of Poritidae (*Porites lutea*), one species of Merulinidae (*Echinopora fruticulosa*) and one hydrozoan (*Millepora dichotoma*). Sailing permits were issued by the Saudi Arabian Coastguard Authority to the sites that included coral collection. Immediately after collection, the coral samples were flash−frozen in liquid nitrogen on board the boat and stored at −80 °C until further processing.

DNA Extraction

For DNA extraction, coral tissues were removed from the skeleton by airblasting with RNase free water using a sterile airbrush (Agora−Tec GmbH, Schmalkalden, Germany). The resulting tissue slurry was collected into sterile 2.0 ml Eppendorf cups, subsequently homogenized and stored at −20 °C until further processing. DNA was extracted from 100 µL of tissue slurry using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany) according to manufacturer's instructions. DNA extraction yields were quantified and qualified using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C until further processing.

Primer selection and quantitative PCR (qPCR)

A total of 18 primers were selected for this study (Table 6.1), that resulted in 10 primer pairs selected for further testing (Table 6.2 and Table 6.3). Primer selection was done by examining the scientific literature for primers that have been used to assess denitrifying microbes preferably performed associated with coral reef organisms or substrates. If unavailable, denitrification primers that have been used on samples from other marine ecosystems were selected. A minimum of two primer pairs targeting functional genes encoding for each enzyme present in the denitrification pathway were selected, i.e. the *narG* gene encoding for nitrate reductase, the *nirK* and *nirS* genes encoding for nitrite reductase, the *norB* gene for

nitric oxide reduction and the *nosZ* gene encoding for nitrous oxide reduction (Table 6.1).

Table 6.1 | Selected primers used for amplification of denitrification genes.

Enzyme	Target gene	Primer[a]	Nucleotide sequence (5' → 3')[b]	Reference
Nitrate reductase	*narG*	narGW9F	MGNGGNTGYCCNMGNGGNGC	Gregory et al.[2]
		narGT38R	ACRTCNGTYTGYTCNCCCCA	
		narG−F	TCGCCSATYCCGGCSATGTC	Bru et al.[3]
		narG−R	GAGTTGTACCAGTCRGCSGAYTCSG	
Nitrite reductase	*nirK*	nirK1F	GGMATGGTKCCSTGGCA	Braker et al.[4]
		nirK5R	GCCTCGATCAGRTTRTGG	
		nirK127R	CCTGCTCACCGACATAATAGA	Zhang et al.[5]
Nitrite reductase	*nirS*	nirS1F	CCTAYTGGCCGCCRCART	Braker et al.[4]
		nirS6R	CGTTGAACTTRCCGGT	
		cd3aF	GTSAACGTSAAGGARACSGG	Michotey et al.[6]
		R3cd	GASTTCGGRTGSGTCTTGA	
Nitric oxide reductase	*norB*	qnorB2F	GGNCAYCARGGNTAYGA	Braker and Tiedje[7]
		qnorB5R	ACCCANAGRTGNACNACCCACCA	
		cnorB2F	GACAAGNNNTACTGGTGGT	
		cnorB6R	GAANCCCCANACNCCNGC	
Nitrous oxide reductase	*nosZ*	nosZ−F	CGYTGTTCMTCGACAGCCAG	Kloos et al.[8]
		nosZ−R	CGSACCTTSTTGCCSTYGCG	
		nosZ1622R	CGCRASGGCAASAAGGTSCG	Throbäck et al.[9]

[a] Forward and reverse primers are indicated by the letters F and R, respectively.

[b] K = G or T; M = A or C; N = A, C, G or T; R = A or G; S = G or C; Y = C or T

A temperature gradient PCR was applied (from 51 °C to 62 °C) to assess the optimal annealing temperature of every primer pair. All PCRs were run in duplicates containing 10 µL of Qiagen Multiplex mix (from the Qiagen Multiplex PCR Kit), 0.5 µM of each primer (10 µM), 1 µL of DNA template and PCR water to adjust the total reaction volume to 20 µL. The thermal cycler protocol was 94 °C for 15 min, followed by 50 cycles of 94 °C for 30 s, 51 to 62 °C for 1 min followed by 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplification products were visually analysed using 1 % agarose gel electrophoresis in 1x TAE buffer.

Quantitative PCR (qPCR) was used to test primers that gave a single or dominant amplicon of the correct size with conventional PCR. qPCR assays were performed in triplicates for each coral replicate. Each assay contained 9 µL reaction mixture and 1 µL DNA template. Reaction mixture contained Platinum SYBR Green qPCR Master Mix (Invitrogen, Carlsbad, CA, United States), 0.2 µL of each primer (10 µM), 0.2 µL of ROX dye and 3.4 µL of RNAse−free water. The thermal cycling protocol was 50 °C for 2 min, 95 °C for 2 min, 50 cycles of 95 °C for 30 s, "optimal annealing temperature" for 1 min, 72 °C for 1 min and a 72 °C extension cycle for 2 min. Amplification specificity was determined by adding a dissociation step. All assays were performed on the ABI 7900HT Fast Real−Time PCR System (Applied Biosystems, CA, USA). The amplification products were visually analysed using 1 % agarose gel electrophoresis in 1x TAE buffer.

Table 6.2 | Amplification results[a] of PCR using a range of primers for the denitrification pathway on four Red Sea corals[b] at different annealing temperatures.

Primer pair	Coral species	51.0 °C	51.9 °C	54.1 °C	57.0 °C	59.8 °C	62.0 °C
narGW9F/narGT38R	Pg	–	–	–	–	–	–
	Ce	–	–	–	–	–	–
	Pv	–	–	–	–	–	–
	Sp	–	–	–	–	–	–
narG−F/narG−R	Pg	–	–	–	–	–	–
	Ce	nsa	nsa	–	–	–	–
	Pv	–	–	–	–	–	–
	Sp	nsa	nsa	–	–	–	–
nirK1F/nirK5R	Pg	nsa	nsa	nsa	nsa	–	–
	Ce	nsa	nsa	nsa	nsa	nsa	nsa
	Pv	nsa	nsa	nsa	–	–	nsa
	Sp	nsa	nsa	nsa	nsa	nsa	nsa
nirK1F/nirK127R	Pg	nsa	nsa	–	–	–	–
	Ce	nsa	nsa	nsa	nsa	nsa	nsa
	Pv	nsa	nsa	nsa	nsa	–	nsa
	Sp	nsa	nsa	nsa	nsa	nsa	nsa
nirS1F/nirS6R	Pg	–	–	–	–	–	–
	Ce	nsa	nsa	nsa	nsa	nsa	–
	Pv	nsa	nsa	nsa	nsa	nsa	–
	Sp	nsa	nsa	nsa	nsa	nsa	–
cd3aF/R3cd	Pg	+	+	+	+	+	–
	Ce	nsa	nsa	–	–	–	–
	Pv	nsa	nsa	–	–	–	–
	Sp	+	+	+	+	+	–
qnorB2F/qnorB5R	Pg	–	–	–	–	–	–
	Ce	–	–	–	–	–	–
	Pv	–	–	–	–	–	–
	Sp	–	–	–	–	–	–
cnorB2F/cnorB6R	Pg	–	–	–	–	–	–
	Ce	nsa	nsa	–	–	–	–
	Pv	nsa	nsa	–	–	–	–
	Sp	–	–	–	–	–	–
nosZ−F/nosZ−R	Pg	nsa	nsa	nsa	nsa	nsa	nsa
	Ce	nsa	nsa	nsa	nsa	nsa	–
	Pv	nsa	nsa	nsa	nsa	nsa	nsa
	Sp	nsa	nsa	–	–	–	–
nosZ−F/nosZ1622R	Pg	nsa	nsa	–	–	–	–
	Ce	nsa	nsa	nsa	nsa	nsa	–
	Pv	nsa	nsa	nsa	nsa	nsa	–
	Sp	nsa	nsa	–	–	–	–

[a] Symbols: Amplicon of the correct size (+); no amplicon (–); multiple amplicons or single amplicon of the wrong size, i.e. no specific amplification (nsa)

[b] Corals are abbreviated as follows: *Pleuractis granulosa* (Pg); *Ctenactis echinata* (Ce); *Pocillopora verrucosa* (Pv); *Stylophora pistillata* (Sp)

6.3 | Results

In the first batch of tested corals, amplicons of the correct size (425 bp) were found in *S. pistillata* and *P. granulosa* with the primer pair cd3aF/R3cd (Figure 6.1 and Table 6.2). Clearest amplicons (as qualified with gel electrophoresis) were found with an annealing temperature of 51 °C (Figure 6.1 and Table 6.2). Results of subsequent qPCRs mirrored results obtained from conventional PCRs. For the second batch of corals the primer pair cd3aF/R3cd was used with an annealing temperature of 51 °C. In addition to *S. pistillata* and *P. granulosa*, amplicons of the correct size were found in *A. hemprichii* and *M. dichotoma* (Figure 6.2 and Table 6.3). Amplification products of subsequent qPCRs mirrored products obtained from conventional PCRs for each sample.

Table 6.3 | Amplification results[a] of PCR using a range of primers for the denitrification pathway on nine Red Sea corals[b].

Primer pair[c]	Amplicon length[d]	Pg	Ce	Pv	Sp	Ah	Ap	Pl	Ef	Md	Control
narGW9F/narGT38R	500 bp	–	–	–	–						–
narG–F/narG–R	173 bp	–	nsa	–	nsa						–
nirK1F/nirK5R	514 – 515 bp	nsa	nsa	nsa	nsa						–
nirK1F/nirK127R	127 bp	nsa	nsa	nsa	nsa						–
nirS1F/nirS6R	890 bp	–	nsa	nsa	nsa						–
cd3aF/R3cd	425 bp	+	nsa	nsa	+	+	nsa	–	nsa	+	–
qnorB2F/qnorB5R	224 – 262 bp	–	–	–	–						–
cnorB2F/cnorB6R	389 bp	–	nsa	nsa	–						–
nosZ–F/nosZ–R	453 bp	nsa	nsa	nsa	nsa						–
nosZ–F/nosZ1622R	415 – 453 bp	nsa	nsa	nsa	nsa						–

[a] Symbols: Amplicon of the correct size (+); no amplicon (–); multiple amplicons or single amplicon of the wrong size, i.e. no specific amplification (nsa), empty cells indicate that the assessment was not performed.

[b] Corals are abbreviated as follows: *Pleuractis granulosa* (Pg); *Ctenactis echinata* (Ce); *Pocillopora verrucosa* (Pv); *Stylophora pistillata* (Sp); *Acropora hemprichii* (Ah); *Acropora pharaonis* (Ap); *Porites lutea* (Pl); *Echinopora fruticulosa* (Ef); *Millepora dichotoma* (Md); Negative control (Control).

[c] Annealing temperature of 51 °C for every primer pair.

[d] Basepairs (bp).

6.4 | Conclusion

Here, we studied the performance of 10 primer pairs targeting five functional genes of the denitrification pathway and encoding for the involved main enzymes. Specifically, the selected primer pairs targeted the denitrification gene clusters *nar*, *nir*, *nor* and *nos*. Based on primer performance for DNA isolated from coral, we selected the primer pair cd3af/R3cd which targets the *nirS* gene encoding for the cytochrome cd_1 nitrite reductase. This primer pair was the only pair to give a dominant band of the correct size for several tested corals. Of note, this primer pair was previously found to perform well with DNA from other marine templates, such as coral rock[10], marine sediments[11], as well as environmental samples from intertidal zones[12], and terrestrial ecosystems[13–15], highlighting its broad coverage.

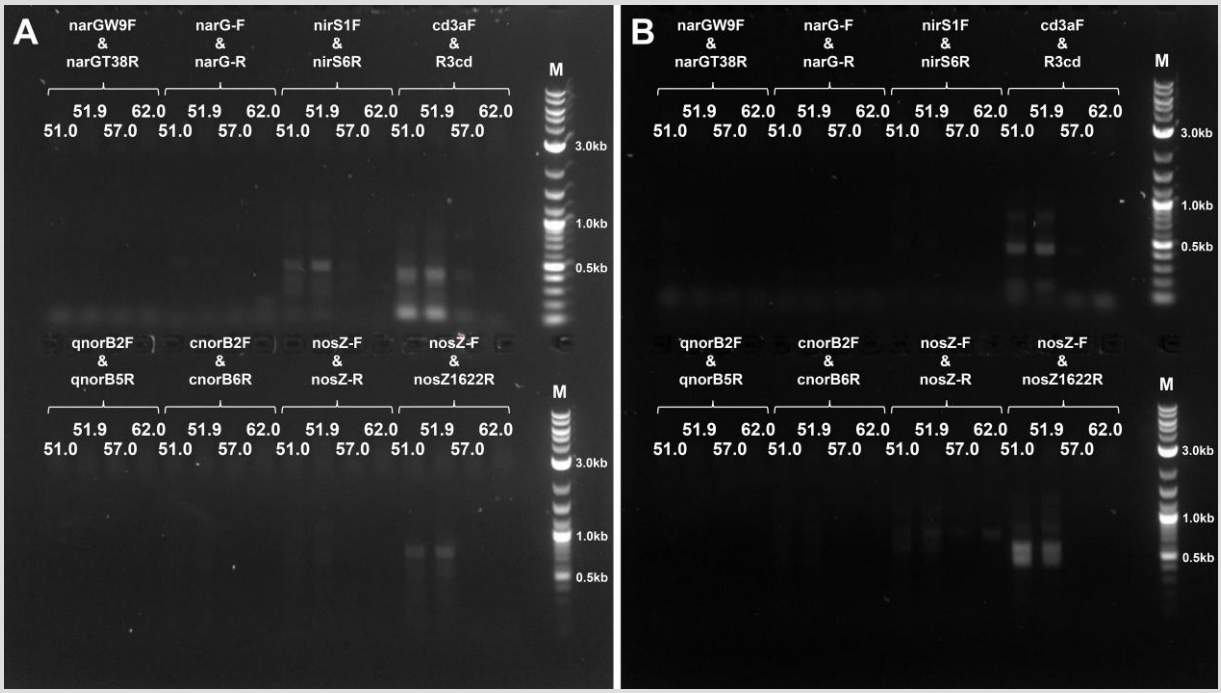

Figure 6.1 | PCR products from eight different primer pairs at four different annealing temperatures for **(A)** *Stylophora pistillata* and **(B)** *Pleuractis granulosa*. Annealing temperatures are 51.0, 51.9, 57.0 and 62.0 °C. M = Marker (2−log DNA ladder). Uncropped gel images originate from two separate gels.

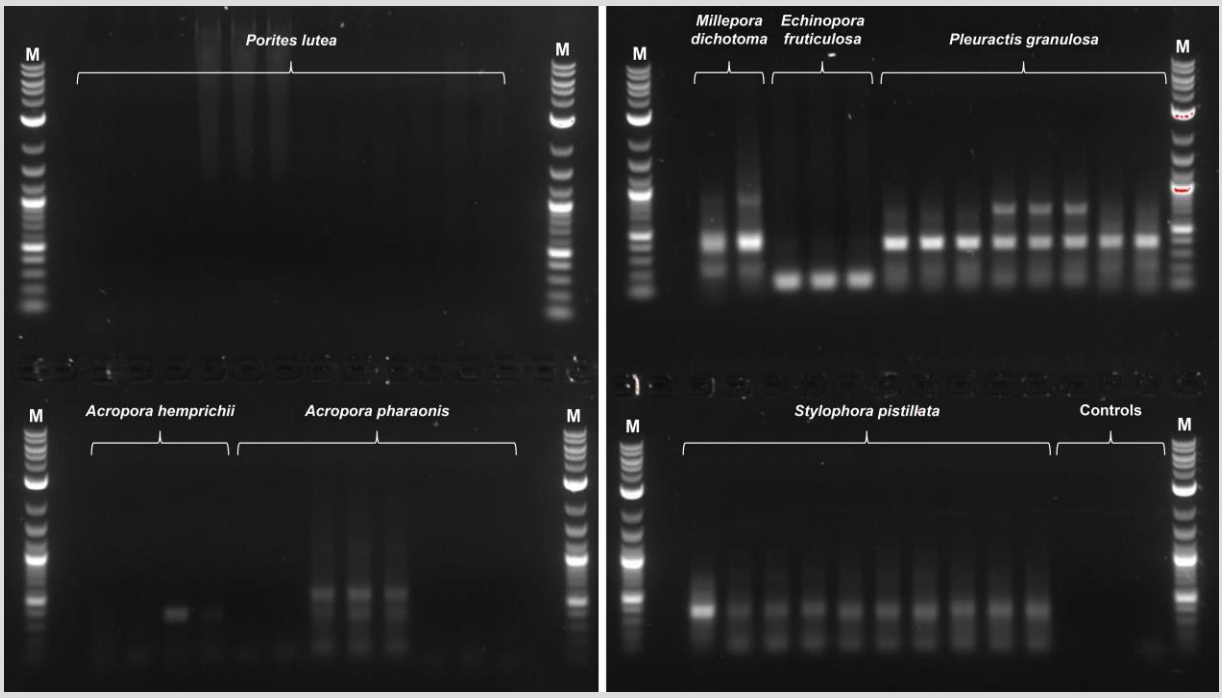

Figure 6.2 | PCR products using the primer pair cd3aF/R3cd at the optimal annealing temperature of 51 °C using 7 different Red Sea corals. M = Marker (2−log DNA ladder). Uncropped gel images originate from two separate gels.

6.5 | References

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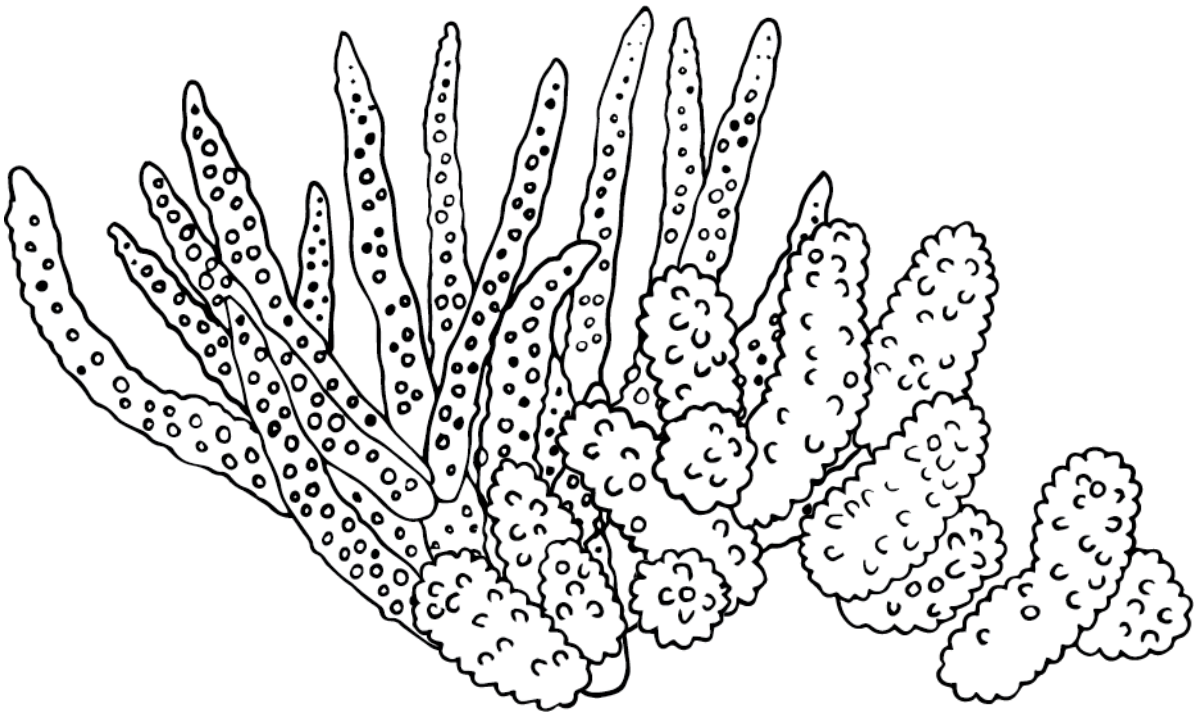
Chapter 7



Chapter 7 | **Evidence for dynamic environmental control of coral holobiont nitrogen cycling**

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7.1 | Abstract

Recent research suggests that nitrogen (N) cycling microbes are important for coral holobiont functioning. In particular, coral holobionts may acquire novel bioavailable N via prokaryotic dinitrogen (N_2) fixation or remove excess N via denitrification activity. However, our understanding of environmental drivers on these processes *in hospite* remains limited. Employing the strong seasonality of the central Red Sea, this study assessed the effects of environmental parameters on the proportional abundances of N–cycling microbes associated with *Acropora hemprichii* and *Stylophora pistillata.* Specifically, we quantified changes in the relative ratio between *nirS* and *nifH* gene copy numbers, as a proxy for seasonal shifts in denitrification and N_2 fixation potential in corals, respectively. In addition, we assessed coral–tissue associated Symbiodiniaceae cell densities and monitored environmental parameters to provide a biotic and environmental context, respectively. Ratios of *nirS* to *nifH* gene copy numbers revealed similar seasonal patterns in both coral species, with ratios closely following patterns in environmental N availability. As Symbiodiniaceae cell densities also aligned with environmental N availability, the seasonal shifts in *nirS* to *nifH* gene ratios are likely driven by N availability in the coral holobiont. Thereby, our results suggest that N–cycling in coral holobionts likely dynamically adjusts to environmental conditions by increasing and/or decreasing denitrification and N_2 fixation potential according to environmental N availability. Microbial N–cycling may, thus, extenuate the effects of changes in environmental N availability on coral holobionts and support maintenance of the coral – Symbiodiniaceae symbiosis.

Keywords: Coral reefs | nitrogen cycling | Scleractinia | seasonality | denitrification | dinitrogen fixation

A modified version of this chapter will be submitted to *Royal Society Open Science*

7.2 | Introduction

The oligotrophic nature of coral reefs requires an efficient use and recycling of the available nutrients within the ecosystem, including their main engineers, scleractinian corals. As such, corals consist not simply of the animal host alone, but additionally harbour a diverse range of eukaryotic and prokaryotic microorganisms[1], rendering it a so–called "holobiont". Many of these coral associated microorganisms aid in nutrient (re)cycling[2,3]. Nitrogen (N) is an essential macro nutrient, the availability of which often being the controlling factor for primary production (i.e. the fixation of inorganic carbon [CO_2] through photosynthesis) in coral holobionts[4,5]. Despite the importance of N for coral holobionts, *in hospite* limitation of N is crucial for maintaining the symbiosis between the coral animal (as the host) and the photosynthetic algal symbionts of the family Symbiodiniaceae[6]. Translocation of photosynthates (derived from Symbiodiniaceae), i.e. the main supply of organic C for the coral host[7], is optimal when Symbiodiniaceae are N limited[8–10]. The interruption of N limitation may thus lead to the cessation of photosynthate translocation, which may ultimately lead to the breakdown of the coral/Symbiodiniaceae symbiosis due to increased bleaching susceptibility [9,11,12]. Thus, the cycling of N is critical for understanding coral holobiont functioning[9].

Environmental availability of N is known to fluctuate in coral reef environments. This may include natural fluctuations, e.g. seasonality in N availability [13–15], as well as anthropogenic N input[16]. In this sense, coral–associated microbes, in particular prokaryotes may play an integral role in coral holobiont N-cycling. On the one hand, diazotrophs, prokaryotes capable of fixing atmospheric dinitrogen (N_2), may provide the coral holobiont with *de novo* bioavailable N in the form of ammonium in times of environmental N scarcity[17–19]. On the other hand, microbes capable of denitrification, i.e. the chemical reduction of nitrate to N_2, may play a putative role in alleviating the coral holobiont from excess N[20]. In that sense, it was hypothesized that high denitrification rates may maintain N limitation for Symbiodiniaceae, and, as a result, may potentially support the functioning of the coral/Symbiodiniaceae symbiosis[9,11]. To this end, the presence of denitrifiers in coral holobionts was first reported in the late 2000s[21,22] and Tilstra et al.[20] recently demonstrated that denitrification indeed constitutes an active metabolic pathway present in coral holobionts from the oligotrophic central Red Sea.

Taken together, microbial N cycling has the potential to increase or reduce N availability for the coral holobiont. However, our understanding of how abiotic and biotic factors affect N-cycling properties in corals remains poorly understood. Making use of the pronounced seasonality of the Red Sea, the present study aimed to (*i*) assess patterns in the abundance of denitrifiers (approximated via *nirS* gene copy numbers) in relation to diazotrophs (approximated via *nifH* gene copy numbers) (from herein referred to as *nirS* to *nifH* gene ratios) in a seasonal resolution; and (*ii*) identify environmental parameters potentially driving the observed seasonal patterns. Due to the potential stimulating or suppressing effects of DIN on denitrification[23] and diazotrophy[24–27], respectively, we hypothesized that the seasonal patterns of *nirS* to *nifH* gene ratios in coral holobionts would be mostly affected by dissolved inorganic N (DIN), i.e. nitrate, nitrite, and/or ammonium concentrations.

7.3 | Materials and Methods

Sample collection

Two common species of hard coral, i.e. *Acropora hemprichii* (Acroporidae) and *Stylophora pistillata* (Pocilloporidae), were collected over four seasons (Figure 7.1A). Corals were collected at approx. 5 m water depth at the semi−exposed side of the inshore reef Abu Shosha (N22°18`15", E39°02`56") located in the Saudi Arabian central Red Sea between April 2017 and January 2018: April 2017 sampling (spring), August 2017 (summer), November 2017 (fall), and January 2018 (winter). During each season, eight fragments of each coral species were collected from spatially separated colonies (> 10 m) to ensure genetic diversity. Immediately after collection, fragments were flash frozen in liquid nitrogen aboard the research vessel. Subsequently, fragments were transported to the laboratories of the King Abdullah University of Science and Technology and stored at −80 °C until further processing.

DNA extraction and quantitative PCR (qPCR)

Quantitative PCRs were carried out according to Tilstra et al.[20]. Briefly, relative copy numbers of the functional genes *nirS* and *nifH* were used as a proxy for denitrification and diazotrophy, respectively, as implemented previously[19,20,28]. To this end, coral tissues were separated from the skeleton by pressurized air. DNA was extracted from 100 µL of the resulting tissue slurry using the Qiagen DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was of varying quality and amplification was not possible in some samples resulting in varying levels of replicates for each species and season.

qPCR assays were performed in triplicates for each biological replicate (i.e., coral fragment). Each assay contained 9 µL reaction mixture and 1 µL DNA template (input adjusted to approx. 3 ng DNA μL^{-1}). Reaction mixture contained 5 µL Platinum SYBR Green qPCR Master Mix (Invitrogen, Carlsbad, CA, United States), 0.2 µL of each primer (10 µM), 0.2 µL of ROX dye and 3.4 µL of RNAse−free water. *NirS* to *nifH* gene ratios were determined by normalizing against the *nifH* gene (see Table 7.1 for primers used). The thermal cycling protocol was 50 °C for 2 min, 95 °C for 2 min, 50 cycles of 95 °C for 30 s, 51 °C for 1 min, 72 °C for 1 min and a 72 °C extension cycle for 2 min. Amplification specificity was determined by adding a dissociation step (melting curve analysis). All assays were performed on the ABI 7900HT Fast Real−Time PCR System (Applied Biosystems, CA, USA). Standard calibration curves were run simultaneously covering 8 orders of magnitude (10^1–10^8 copies of template per assay each for the *nirS* and *nifH* gene). The qPCR efficiency (E) of the primer pairs was 86 % and 87 %, respectively, calculated according to the equation $E = [10^{(-1/slope)}-1]$. *NirS* to *nifH* gene ratios were calculated as $2^{(-\Delta\Delta Ct)}$ against *nifH* Ct values using the season with the lowest relative abundances as the reference[29].

Table 7.1 | Selected primers used for amplification

Target gene	Primer	Nucleotide sequence (5' → 3')	Reference
nirS	cd3aF	GTSAACGTSAAGGARACSGG	Michotey et al.[30]
	R3cd	GASTTCGGRTGSGTCTTGA	
nifH	F2	TGYGAYCCIAAIGCIGA	Gaby and Buckley[31]
	R6	TCIGGIGARATGATGGC	

Symbiodiniaceae cell density

An aliquot of the tissue slurry used for DNA extraction was used to obtain cell densities of Symbiodiniaceae. Tissue slurry aliquots were homogenized, diluted at a ratio of 5:1, and Symbiodiniaceae cells were subsequently counted using a Neubauer−improved hemocytometer on a light microscope with HD camera (Zeiss, Germany). Resulting photographs were analyzed using the Cell Counter Notice in ImageJ software (National Institutes of Health, USA). Cell counts for tissue slurries for each individual coral were done in duplicates and subsequently averaged. Finally, to obtain cell densities of Symbiodiniaceae per unit area of coral tissue, cell counts were normalized to coral surface area, which was calculated using cloud−based 3D models of samples (Autodesk Remake v19.1.1.2)[32,33].

Environmental parameters

Environmental data, i.e. temperature, light intensity (photosynthetically active radiation [PAR = 400–700 nm], salinity, dissolved oxygen, nitrate, nitrite, ammonium, dissolved inorganic phosphorus (DIP = [phosphate]), and dissolved organic carbon (DOC), were described and published previously in Roth et al.[14] and were reanalysed for the purpose of the present study.

Temperature was measured continuously with data loggers (Onset HOBO Water Temperature Pro v2 Data Logger – U22–001; accuracy: ±0.21 °C) one month prior and within the month of sampling on a 30 min interval. Light availability (lux) was measured with data loggers (Onset HOBO Pendant UA– 002–64; spectral detection range 150–1200 nm) for three full days every month and converted to photosynthetically active radiation (PAR = 400–700 nm) using a conversion factor of 51.8. Salinity was measured for three full days every month using a conductivity measuring cell (TetraCon®, 925, WTW, accuracy: ± 0.5 % of value, internal conversion to salinity). Dissolved oxygen (DO) was quantified on 2 days within the month of sampling by taking the average of eight autonomous recording DO and temperature sensors (HOBO U26; temperature corrected and salinity adjusted) that were deployed at 5 m water depth within a radius of 50 m of the sampling site. Seawater samples were taken in triplicates at the sampling site on three days during each month, i.e. one month prior to sampling and the month of sampling, to measure (in)organic nutrients. Nitrate, nitrite and DIP were measured photometrically, while ammonium was measured fluorometrically. DIN:DIP ratios were calculated, where DIN = [nitrate] + [nitrite] + [ammonium]. Subsamples for dissolved organic carbon (DOC) were filtered through 0.2 µm Millipore® polycarbonate filters into pre–combusted (450°C, 4.5 h) acid–washed amber glass vials (Wheaton) with Teflon–lined lids, and samples were subsequently acidified with H_3PO_4 until reaching a pH 1 – 2. Samples were kept in the dark at 4 °C until further analysis by high–temperature catalytic oxidation (HTCO) using a total organic carbon analyzer (Shimadzu, TOC–L). To monitor the accuracy of DOC concentration measurements, we used reference material of deep–sea carbon (42 – 45 µmol C L^{-1}) and low carbon water (1 – 2 µmol C L^{-1}).

Statistical analyses

To assess seasonality, data were analyzed using non−parametric permutational multivariate analysis of variance (PERMANOVA) using PRIMER−E version 6 software[34] with the PERMANOVA+ add on[35]. To test for differences in *nirS* and *nifH* gene ratios and Symbiodiniaceae cell densities between seasons, 2−factorial PERMANOVAs were performed with season and coral species as main factors, while 1−factorial PERMANOVAs were performed with season as a main factor for environmental parameters, based on

Bray–Curtis similarities of square–root transformed data. Type III (partial) sum of squares were therefore used with unrestricted permutation of raw data (999 permutations), and PERMANOVA pairwise tests with parallel Monte Carlo tests were carried out when significant differences were found.
Pearson Product–Moment Correlation tests were performed to identify correlations between *nirS* to *nifH* gene ratios, Symbiodiniaceae cell density and environmental variables. Salinity and nitrite were omitted from the analyses as differences were assumed to have no ecological significance. Finally, linear regression analysis was used to assess a potential statistical relationship between *nirS* to *nifH* gene ratios and Symbiodiniaceae cell density over all seasons. All values are given as mean ± SE.

7.4 | Results

NirS *to* nifH *gene ratios*

Lowest *nirS to nifH* gene ratios were observed during the spring season, hence gene ratios for other seasons were calculated as fold changes in relation to spring (Figure 7.1B). In the summer, gene ratios increased ~2–fold for both species, while they increased ~11–fold during the fall for *A. hemprichii* and ~52–fold for *S. pistillata* (Figure 7.1B). During winter, gene ratios were ~5–fold higher in *A. hemprichii* compared to spring (no data available for *S. pistillata* for this season) (Figure 7.1B). Due to strong variation there were no significant differences found between seasons for *A. hemprichii*. For *S. pistillata* gene ratios were higher during fall compared to spring (pair–wise PERMANOVA, t = 2.83, p = 0.038). There was no interactive effect of season and species on gene ratios (PERMANOVA, pseudo–F = 0.14, p = 0.877; Supplementary Table S7.1). However, there was an effect of season (PERMANOVA, pseudo–F = 3.04, p = 0.039; Supplementary Table S7.1) and species (PERMANOVA, pseudo–F = 6.11, p = 0.019; Supplementary Table S7.1) on gene ratios. Indeed, gene ratios were higher during fall compared to spring (pair–wise PERMANOVA, t = 3.12, p = 0.013) and summer (pair–wise PERMANOVA, t = 2.45, p = 0.024).

Symbiodiniaceae cell density

Cell densities of Symbiodiniaceae varied more strongly between seasons in *A. hemprichii* compared to *S. pistillata* (Supplementary Table S7.1; Figure 7.1C). Cell densities for *A. hemprichii* were lowest in spring (0.31 ± 0.03 x10^6 cells cm^{-2}) and significantly increased during summer (0.56 ± 0.05 x10^6 cells cm^{-2}; pair–wise PERMANOVA, t = 4.14, p < 0.001) (Figure 7.1C). Subsequently, cell densities significantly increased in fall (0.78 ± 0.06 x10^6 cells cm^{-2}; pair–wise PERMANOVA, t = 2.69, p = 0.014), but returned to densities similar to summer, during winter (0.59 ± 0.10 x10^6 cells cm^{-2}) (Figure 7.1C). Cell densities of Symbiodiniaceae in tissues of *S. pistillata* were similar during spring, summer and winter (0.53 ± 0.07, 0.52 ± 0.08, 0.50 ± 0.04 x10^6 cells cm^{-2}, respectively) (Figure 7.1C). However, densities during fall were significantly higher compared to the other seasons (0.73 ± 0.06 x10^6 cells cm^{-2}; pair–wise PERMANOVA, p < 0.05) (Figure 7.1C).

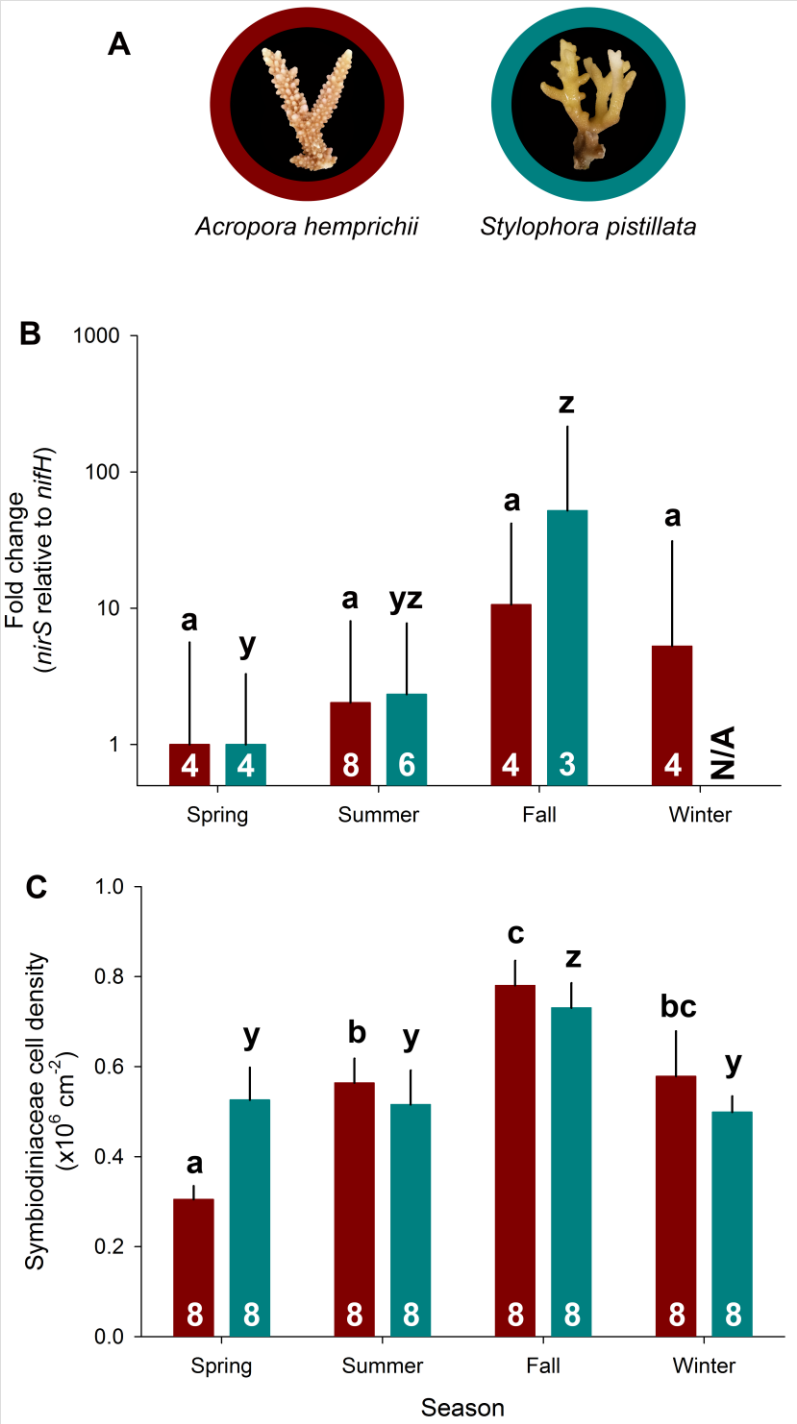

Figure 7.1 | Patterns of *nirS* to *nifH* gene ratios and Symbiodiniaceae cell densities associated with two Red Sea hard coral species across four seasons. **(A)** Representative photographs of investigated species, **(B)** Fold change of *nirS* to *nifH* gene ratios, **(C)** Symbiodiniaceae cell densities. Fold changes were calculated in relation to spring, during which both species exhibited the lowest *nirS* to *nifH* gene ratios; bars indicate the mean; error bars indicate upper confidence intervals (+ 1 SE). Numbers in the bars represent the sample size (*n*). Different letters above error bars indicate statistically significant differences per species between seasons within each plot (pair–wise PERMANOVA, $p < 0.05$). N/A = not available.

Environmental parameters

Several environmental parameters exhibited marked seasonal fluctuations (Figure 7.2). Temperature and PAR increased from spring to the summer season when both parameters were at their highest (31.99 ± 0.01 °C and 573 ± 13 µmol m^{-2} s^{-1}, respectively) (Figure 7.2A and Figure 7.2B). Dissolved oxygen was lowest in summer (5.26 ± 0.05 mg L^{-1}) and highest in winter (6.44 ± 0.03 mg L^{-1}) (Figure 7.2C). Nitrate was highest during the fall season (0.93 ± 0.02 µM) and lowest during the spring season (0.30 ± 0.05 µM) (Figure 7.2D). Nitrite remained stable throughout all seasons (0.045 ± 0.005 µM) (Figure 7.2E). Ammonium was highest during summer (0.19 ± 0.03 µM) (Figure 7.2F). DIN followed the same pattern as nitrate being highest during the fall season (1.11 ± 0.03 µM) and lowest during the spring season (0.46 ± 0.08 µM) (Figure 7.2G). DIP was stable from spring until fall but decreased during winter (0.08 ± 0.01 µM) (Figure 7.2H). Salinity remained relatively stable throughout the period of study (39.85 ± 0.015 PSU) (Figure 7.2I). DOC followed the same seasonal pattern as temperature, being highest in summer (77.36 ± 0.47 µM) and lowest in spring (67.44 ± 0.27 µM) and winter (68.99 ± 0.77 µM) (Figure 7.2J).

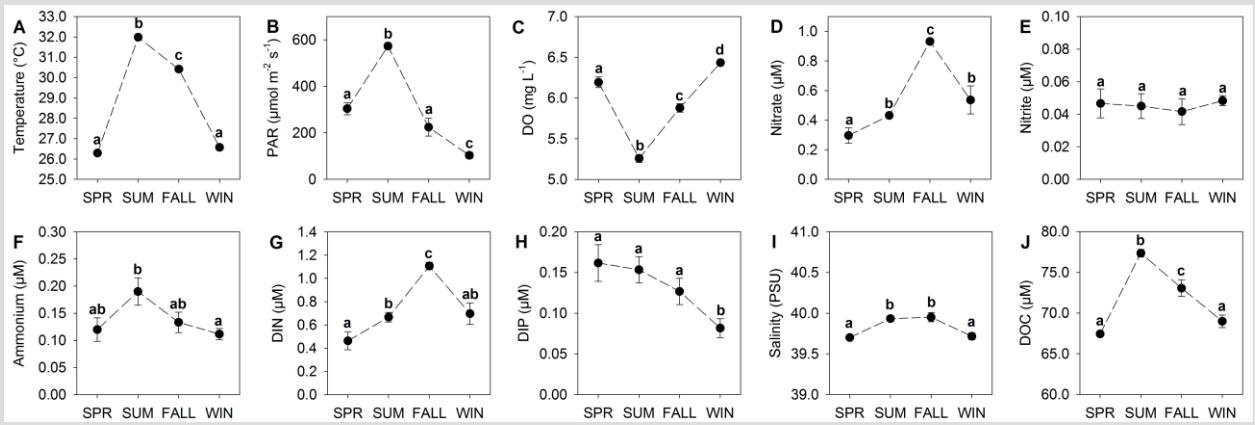

Figure 7.2 | Means (±S.E.) of environmental parameters measured over four seasons. **(A)** Temperature, **(B)** Photosynthetically Active Radiation (PAR), **(C)** dissolved oxygen (DO), **(D)** nitrate**, (E)** nitrite, **(F)** ammonium, **(G)** Dissolved Inorganic Nitrogen (DIN = [nitrate] + [nitrite] + [ammonium]), **(H)** Dissolved Inorganic Phosphorus (DIP = [phosphate]), **(I)** salinity, **(J)** Dissolved Organic Carbon (DOC). Different letters above error bars indicate significant differences between seasons within each plot ($p < 0.05$). SPR = spring; SUM = summer; WIN = winter. Data were extracted from Roth et al.[14] and re–analysed for the purpose of this study.

Correlation analyses

Due to the lack of a significant interaction between season and species *nirS to nifH* gene ratios (Supplementary Table S7.1), data for both species were pooled for correlation analyses.

The strongest correlation for both species' *nirS to nifH* gene ratios was with nitrate (Pearson Product–Moment Correlation, r = 0.463, $p = 0.007$; Figure 7.3A; Supplementary Table S7.2). Symbiodiniaceae cell densities also correlated strongest with nitrate for both *A. hemprichii* (Pearson Product–Moment Correlation, r = 0.649, $p < 0.001$; Figure 7.3B; Supplementary Table S7.2) and *S. pistillata* (Pearson Product–Moment Correlation, r = 0.446, $p = 0.011$; Figure 7.3C; Supplementary Table S7.2).

No relationship was found between *nirS to nifH* gene ratios and Symbiodiniaceae cell densities for *A. hemprichii* (linear regression, F = 1.35, $r^2 = 0.07$, $p = 0.260$) and *S. pistillata* (linear regression, F = 2.21, r^2 =

0.17, $p = 0.165$).

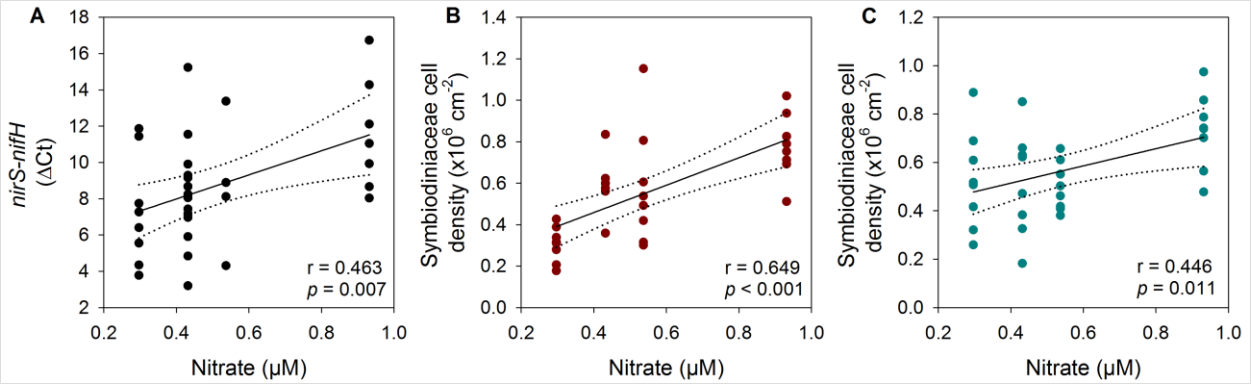

Figure 7.3 | Pearson Product–Moment Correlation analyses for **(A)** ΔCt of *nirS–nifH* against environmental nitrate concentrations pooled for both coral species, and cell densities of Symbiodiniaceae against environmental nitrate concentrations for **(B)** *Acropora hemprichii* and **(C)** *Stylophora pistillata*. r = Pearson coefficient. Dotted lines represent 95% confidence intervals.

7.5 | Discussion

Coral associated microbial N–cycling still remains an understudied, but potentially very important part of coral holobiont functioning as it may be a source or sink of bioavailable N[9]. Here, we assessed the proportional dynamics of two antagonistic N–cycling pathways, i.e. denitrification and diazotrophy, in two common central Red Sea coral species (Figure 7.1A) in a seasonal resolution. To this end, proportional abundances of the functional marker gene *nirS*, as a proxy for denitrification[20], were calculated in relation to the functional marker gene *nifH*, as a proxy for diazotrophy[19] (Figure 7.1B). Importantly, the *nirS to nifH* gene ratios presented in this study are not based on absolute, but relative abundances of each respective marker gene. Consequently, the here presented approach does not allow for any conclusion regarding the absolute abundance of marker genes, or, ultimately, absolute abundances of either denitrifiers or diazotrophs. Rather, changes in the ratio may be interpreted as a proxy for a shift in the relative abundance of denitrifying in relation to N_2–fixing prokaryotes. In this light, increasing ratios may reflect an increase in denitrifying microbes and/or a decrease in N_2–fixing microbes and *vice versa*. Using this approach, we were able to reveal the seasonal dynamics of microbial nitrogen cycling in Red Sea corals.

Seasonal patterns and environmental drivers of denitrification and N_2 fixation potential in corals

The *nirS to nifH* gene ratios followed a very similar pattern in both coral species across seasons (Figure 7.1B). Fall was characterized by the highest and spring by the lowest *nirS to nifH* gene ratios in both species. While we did not provide measurements of pathway activity in the present study, relative gene abundances of both marker genes (i.e. *nirS* and *nifH*) were previously shown to align with denitrification and N_2 fixation rates, respectively[19,20]. Consequently, the observed patterns of *nirS to nifH* gene ratios will likely translate into similar seasonal patterns for associated denitrification to N_2 fixation activities. In this light, the similarity of the seasonal patterns found in both coral species suggests that the functional niche occupied by different N–cycling microbes may be very similar and highly responsive to changing environmental conditions.

Among all investigated environmental parameters, nitrate (the biggest contributor to DIN throughout all four investigated seasons; Supplementary Figure S7.1) and DIN concentrations showed the strongest correlation with relative *nirS* to *nifH* gene ratios across coral species (Supplementary Table S7.2). As the substrate for denitrifiers, nitrate may directly stimulate denitrification activity[23]. Likewise, increased nitrate and/or ammonium concentrations have been shown to depress diazotroph activity[24–27]. The observed patterns in relative *nirS to nifH* gene ratios may, thus, be the direct consequence of increased environmental N availability in the coral holobiont.

The notion of seasonally changing N availability driving patterns in ratios of prokaryotic N–cycling functional groups within coral holobionts is corroborated by the patterns of Symbiodiniaceae cell densities observed in both coral species. Similar to *nirS to nifH* gene ratios, Symbiodiniaceae cell densities exhibited strong seasonal differences that positively correlated with environmental nitrate and DIN concentrations (Figure 7.3B and Figure 7.3C, Supplementary Table S7.2). As Symbiodiniaceae population densities are known to be governed by N availability in the stable coral–algae symbiosis[36,37], this suggests that environmental N availability was closely linked with N availability within the coral holobiont in the present study as previously observed in *ex situ* studies[38–40].

Dynamics of N–cycling microbes as a buffer against seasonal changes in environmental N availability?

Limited N availability is critical to coral holobiont functioning as it limits population growth of Symbiodiniaceae *in hospite* and maintains high rates of translocation of photosynthetic carbon (C) to the host[8–10,41]. Seasonal or anthropogenically driven increases in environmental N availability may consequently stimulate Symbiodiniaceae proliferation, thereby disrupting or reducing organic C translocation to the host, ultimately posing a threat to overall coral holobiont functioning[11,42]. Yet, coral holobionts manage to thrive in highly dynamic environments with considerable temporal and spatial variations in N availability[13–15]. The positive correlation of Symbiodiniaceae densities and environmental N availability in the present study suggests that the coral hosts may not have been able to fully maintain stable N availability within the holobiont. In this light, the observed increase in relative *nirS to nifH* gene ratios with increasing N availability, suggests an increase in denitrifying prokaryotes, likely reflecting a beneficial role of N-cycling microbes in regulating N availability within the holobiont. During periods of low N availability (e.g., spring), low relative *nirS to nifH* gene ratios likely implies reduced denitrification and increased N_2 fixation activity. Likewise, during periods of high N availability (e.g., fall), high relative *nirS to nifH* gene ratios likely reflect increased denitrification and reduced N_2 fixation activity. If indeed translatable to corresponding prokaryotic activity, the observed dynamics in functional N cycling gene ratios may, thus, directly support coral holobiont functioning[9]. Specifically, the interplay of denitrifiers and N_2–fixers may support the removal of excess N during times of excess N availability, whilst providing access to new bioavailable N in times of low environmental N availability[9,17]. Whilst these processes may evidently have been insufficient to the stabilization of N availability within the holobiont as shown in the present study, they may be directly assisting the coral host in regulating Symbiodiniaceae populations.

Future research directions

The present study adds to a rapidly growing body of research highlighting the functional importance of N–cycling microbes in coral holobiont functioning. Deciphering the interactions between N–cyclers and other coral holobiont members promises to advance our understanding of coral holobiont functioning in light of

environmental conditions and anthropogenically–driven change. Whilst combined molecular (sequencing, real–time PCR) and physiological approaches haven proven powerful tools to study N–cycling properties of coral holobionts, future studies should aim to address the localization of the main microbial players along with an accurate quantification of metabolic interactions with other holobiont members. In this light, fluorescence *in situ* hybridization (FISH) as well as nanoscale secondary ion mass spectrometry (NanoSIMS) techniques may allow for an integrated and functional understanding of metabolic interactions in light of their localization within the coral holobiont.

7.6 | Acknowledgements

We thank KAUST CMOR staff and boat crews for their support with diving operations. Financial support was provided by KAUST baseline funds to C.R. Voolstra and the German Research Foundation (DFG) grant Wi 2677/9−1 to C. Wild.

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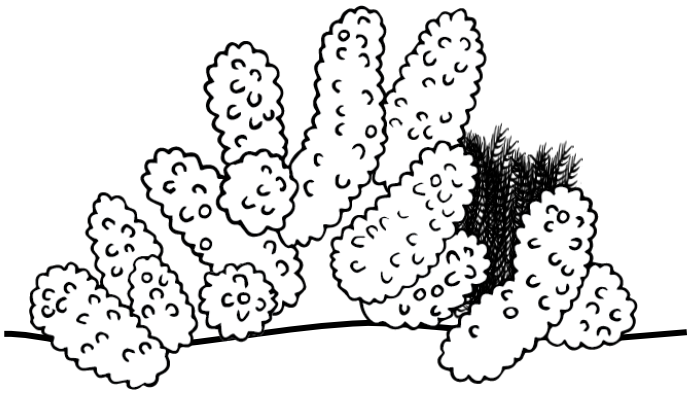
Chapter 8



Chapter 8 | *In situ* eutrophication stimulates dinitrogen fixation, denitrification and productivity in Red Sea coral reefs

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8.1 | Abstract

Eutrophication (i.e. the increase of (in)organic nutrients) may affect functioning of coral reefs, but knowledge about the effects on nitrogen (N) cycling and its relationship to productivity within benthic reef communities is scarce. We thus investigated how *in situ* manipulated eutrophication impacted productivity along with two counteracting N–cycling pathways (dinitrogen (N_2) fixation, denitrification), using a combined acetylene assay. We hypothesised that N_2 fixation would decrease, and denitrification increase in response to eutrophication. N fluxes and productivity (measured as dark and light oxygen fluxes assessed in incubations experiments) were determined for three dominant coral reef functional groups (reef sediments, turf algae, and the scleractinian coral *Pocillopora verrucosa*) after eight weeks of *in situ* nutrient enrichment in the central Red Sea. Using slow–release fertiliser, we increased the dissolved inorganic N concentration by up to 7–fold compared to ambient concentrations. Experimental nutrient enrichment stimulated both N_2 fixation and denitrification across all functional groups compared by 2– to 7–fold, and 2– to 4–fold, respectively. Productivity doubled in reef sediments and remained stable for turf algae and *P. verrucosa*. Our data therefore suggest that (*i*) turf algae are major N_2–fixers in coral reefs, while denitrification is widespread among all investigated groups; (*ii*) surprisingly and against hypothesis both N_2 fixation and denitrification are involved in processing moderate N eutrophication, and (*iii*) stimulated N_2 fixation and denitrification are not directly influenced by productivity. Our findings underline the importance and ubiquity of microbial N cycling in (Red Sea) coral reefs along with its sensitivity to eutrophication.

Keywords: Nitrogen cycle | climate change | pollution | Red Sea | acetylene reduction assay | Acetylene inhibition assay

A modified version of this chapter has been accepted for publication in *Marine Ecology Progress Series* https://doi.org/10.3354/meps13352

8.2 | Introduction

Coastal zones have always attracted humans for various reasons (e.g., resources, transportation and logistics, recreational activities), and migration to coastal cities and areas is increasing[1]. Eutrophication is one of many stressors that intensifies congruently to growing coastal populations[2]. Coastal nutrient point–sources, i.e., excessive nutrient loads from (un)controlled sewage dumping or agricultural fertiliser runoff, play a major role in reshaping nearshore ecosystems such as coral reefs[3,4]. Coral reefs, however, are usually adapted to low nutrient (i.e., oligotrophic) environments, but paradoxically belong to the most diverse and productive ecosystems on earth[5] with nitrogen (N) acting as an important factor limiting productivity[6].

Determining the effects of excessive nutrient availability on tropical coral reefs has been part of numerous studies in the last decades displaying beneficial as well as negative effects. It has been demonstrated that an increase in inorganic matter can shift roles of heterotrophy and autotrophy in coral energetics[7], and that high loads of particulate organic matter do not always negatively impact on the physiology of corals[8]. Furthermore, enhanced heterotrophic feeding on zooplankton can facilitate coral tissue growth and calcification rates[9], whereas coral starvation can lead to lower photosynthetic activity, congruently with lower lipid and protein concentrations[10]. Investigations of long–term nutrient enrichment effects revealed threats on coral reefs on multiple levels, ranging from a higher susceptibility of corals to bleaching and mortality[11,12], to negative effects on coral growth and calcification rates[13,14] or reproductive success[15,16]. When nutrient availability increases over extended periods, phase shifts from coral–dominated reefs to (macro)algae–dominated states of a reef are likely to occur[17]. Moreover, nutrient enrichment together with other anthropogenic stressors that haven proven to alter N–cycling processes, such as ocean warming[18] or ocean acidification[19,20], may result in synergistic effects that ultimately decrease reef resilience and eventually lead to reef degradation or reef losses[17].

For a better understanding of ecosystem functioning in general, and the effects of elevated nutrients on ecosystem functioning in particular, studying N–cycling in coral reefs is of paramount interest. The import of *de novo* bioavailable N to the system is partly performed by diazotrophs, i.e. microbes capable of fixing atmospheric dinitrogen (N_2) into bioavailable ammonium (NH_4^+). This N_2 fixation is crucial for coral reef ecosystems to maintain the N supply and satisfy N demands[6,21]. N_2 fixation rates in coral reefs can fluctuate seasonally and with response to variation in environmental conditions[22]. At the same time, microbial denitrification removes bioavailable N from the ecosystem as it facilitates the reduction of nitrate (NO_3^-) to N_2 and can, thus, be described as a counteracting pathway to N_2 fixation[23,24]. However, knowledge about denitrification in coral reef environments is scarce. Hypothetically, denitrification is vital to coral reef ecosystem functioning, especially under eutrophic conditions as this process removes excess N from the reef system[14,25].

N–cycling activity in coral reefs depends on the environmental nutrient availability[14,22] that can naturally be shaped by upwelling[26], terrestrial runoff after rainfalls[27] or by anthropogenic sources[16,28]. As such, N–cycling in coral reef environments has the potential to exacerbate or attenuate eutrophication events. However, little is known about N–cycling in coral reefs under elevated nutrient availability. We hypothesised significant responses of N_2 fixation and denitrification rates to eutrophication[29]. In case of N_2 fixation, we expect decreasing activity in eutrophic environments as hypothesised before[30]. Koop et al.[14] were able to demonstrate this in coral reef–associated sediments of the Great Barrier Reef (GBR). This phenomenon was attributed to the idea that bioavailable N, e.g. in the form of NH_4^+ or NO_3^-, offers a more cost–efficient, alternative source of N to an organism. Furthermore, the presence of fixed N potentially

inhibits the enzyme (nitrogenase) activity responsible for N_2 fixation[31,32]. For denitrification, we expected an increase with increasing nutrient availability in accordance to other studies[25,29] to subsequently sustain favourable N–limitation[11]. Overall, we anticipate a dynamic interplay of N_2 fixation and denitrification which counterbalances changes in environmental dissolved inorganic nitrogen (DIN) availability.

We here aimed to assess the effects of elevated nutrient availability on N–cycling in a coral reef. We used an eight–week *in situ* nutrient manipulation experiment in natural reef communities in the oligotrophic central Red Sea to investigate the effects of eutrophication on several metabolic processes (N_2 fixation, denitrification, respiration, and photosynthesis) in a comparative framework with three major functional groups (scleractinian coral, filamentous turf algae, and carbonate reef sediments).

8.3 | Materials and Methods

Experimental design

The in-situ manipulation experiment was conducted from late January until late March 2018 in a semi–exposed area of the Abu Shoosha reef in the Jeddah Region (22° 18` 15” N, 39° 02` 56” E) on the west coast of Saudi Arabia in the central Red Sea. Eight distinct (i.e., > 5 m apart from each other) natural reef communities at a water depth of 5–6 m were chosen. Four slow–release fertiliser tubes (Osmocote Plus (15–9–12)) were attached with pins around each reef community (Figure 8.1). Osmocote Plus fertiliser supplied various macronutrients non–stop (15 % total N, 9 % available phosphate (P), 12 % soluble potash, a detailed list of released micronutrients can be found in Supplementary Table S8.1) from the 1st day of fertilisation under local temperature regimes[33], and has been successfully utilised in previous eutrophication studies[34,35]. As nutrients tend to leach out during the first weeks[33] and to reduce resulting bias, fertiliser pins were renewed every two weeks to ensure continuous nutrient inputs. To account for dilution effects with surrounding waters, water samples from different distances to the fertiliser were taken every second week to quantify nutrient concentrations. Specimens of target organisms and substrates were taken from close radius (max. 25 cm) from the fertiliser. The eutrophication phase lasted for eight weeks and specimens of the fertilised communities (hereafter “eutrophied communities”) were taken at the end of the manipulation phase. Specimens from the surrounding, non–fertilised reef communities (hereafter “control communities”) were taken for comparative analysis at the same time.

Figure 8.1 | Manipulated *in situ* community. Four pins with attached fertiliser bags. Photo: Florian Roth.

Collection and maintenance

We tested three of the most dominant biotic and abiotic functional groups of benthic reef communities of the Central Red Sea (carbonaceous reef sediments, filamentous turf algae, and the scleractinian coral – *Pocillopora verrucosa*). These three groups contribute to more than 70 % of the benthic community composition of the sampled reef[36]. Sediments were collected using a Petri dish (material: polystyrene; diameter: 5.5 cm, height: 1.4 cm) that was pushed carefully into the sediment. Sediments were then fixed to the dish from underneath so that upper sediment "cores" with a max. sediment depth of 14 mm were sampled. Turf algae were defined as dead coral fragments of approx. 10 cm length, overgrown with dense and flat (< 2 cm in height) assemblages of filamentous algae of different species, including small individuals of macroalgae and cyanobacteria. Examples from Northern Red Sea studies show that turf algae account for the highest fraction (up to 90 %) of benthic algal cover[37]. Fragments were collected with hammer and chisel. *P. verrucosa* fragments were approx. 10 cm long and were collected with the same tools from different coral colonies to ensure genetic variability. Coral colonies had a minimum of 8–10 m distance between each other. All fragments and Petri dishes containing reef sediment samples were immediately transferred to recirculation aquaria on the boat after sampling (n = 4 from eutrophied communities into aquaria each filled with 10 L of 5 µM NO_3 enriched seawater; n = 5 from control communities into ambient seawater; NO_3 enrichment consisted of previously prepared $NaNO_3$ stock solution, prepared with MilliQ water and $NaNO_3$, ≥ 99.0 %, Sigma–Aldrich) and kept at ambient water temperature and light conditions until the experimental incubations started within 3 h after sampling.

Environmental parameters

Key environmental parameters were assessed every second week throughout the total manipulation period of eight weeks. Details for analytical approaches are described in Roth et al.[36]. Briefly, seawater temperature was measured continuously with data loggers (Onset HOBO Water Temperature Pro v2 Data Logger – U22–001; accuracy: ± 0.021 °C). Seawater samples for assessing concentrations of NO_3^-, nitrite (NO_2^-), and phosphate (PO_4^{3-}) were taken from various distances from fertiliser pins (i.e., directly at the fertiliser pins, 25 cm from inside the communities, > 200 cm outside the communities serving as controls). Water samples were filtered immediately on the boat (Isopore™ membrane filters, 0.2 µm GTTP) and the filtrate was stored at 4 °C in the dark until frozen at –50 °C in the lab within 3 h after collection. Nutrient concentrations were determined using a continuous flow analyser (AA3, HR, SEAL), following colourimetric standard methods[38]. Limits of quantification (LOQ) for NO_3^-, NO_2^- and PO_4^{3-} were 0.084 µmol L^{-1}, 0.011 µmol L^{-1}, and 0.043 µmol L^{-1} respectively. From these seawater samples, 5 mL subsamples were taken for NH_4^+ determination using the ortho–phthaldialdehyde (OPA) method[39,40]. Samples were filtered into separate acid–washed centrifuge tubes, and 1.2 mL OPA solution was added. Samples were then stored for > 4 h in the dark. NH_4^+ was determined fluorometrically within 8 h (Trilogy® Laboratory Fluorometer, Turner Designs Inc.). The LOQ for NH_4^+ was 0.094 µmol L^{-1}. NO_3^-, NO_2^- and NH_4^+ were measured in combination, termed DIN, and presented in [mean ± standard error of mean µM N] hereafter.

Primary production, N_2 fixation and denitrification measurements

Incubations were conducted *ex situ* and < 3 h after sample collection. For oxygen (O_2) flux measurements, incubation chambers (1 L volume) were filled exclusively with ambient seawater collected the same day (n = 5, with specimens from control communities), and four incubation chambers were filled with seawater

and amended with 5 µM NO_3 to provide and keep eutrophic conditions (n = 4, with specimens from eutrophic communities). Additionally, two chambers without specimens (one filled with seawater, one filled with 5 µM NO_3 enriched seawater) served as controls to correct for planktonic background metabolism. All chambers were sealed gastight and without any air enclosure. During the incubations, the incubation chambers were placed in a tempered water bath and constantly stirred (500 rpm) to ensure stable measurement conditions (27 °C). A 2 h light (photon flux of ~200 µM quanta m^{-2} s^{-1}) incubation was followed by a 2 h dark incubation with fresh ambient and nutrient–enriched seawater, respectively. O_2 levels were measured immediately before starting the respective incubations and after 2 h using a WTW Multi 3430 which was equipped with a WTW DFO 925 oxygen sensor. Measured values from dark and light incubations were used to calculate dark respiration (hereafter *R*) and net primary production (hereafter P_n): O_2 start concentrations were subtracted from end concentrations and results were then normalised to incubation time. In the next step, O_2 fluxes were corrected for the seawater control signal, related to incubation volume and normalised to the surface area of the organisms/substrates. Surface areas for turf algae fragments and *P. verrucosa* were calculated using cloud–based 3D models of samples (Autodesk Remake v19.1.1.2)[41]; Gutierrez–Heredia et al., 2016). Surface areas of sediments were mathematically calculated (Surface area = π * radius^2) as Petri dishes were used for sampling sediment cores. Subsequently, gross primary production (hereafter P_g) rates were calculated according to $P_g = P_n - |R|$.
N_2 fixation and denitrification incubations were performed using a COmbined Blockage/Reduction acetylene Assay (COBRA) with the same specimens 3 to 4 h after the O_2 flux measurements. The incubations were performed as described previously in El–Khaled et al.[42]. Briefly, all COBRA incubations were conducted in gas–tight 1 L glass chambers, each filled with 800 mL of nutrient–enriched seawater (5 µM NO_3) and 200 mL headspace. Nutrient–enriched seawater was used in all treatments, as acetylene inhibits the production of NO_3^- in the nitrification pathway[43]. As NO_3^- serves as a substrate for denitrification, the inhibition of nitrification potentially results in an underestimation of denitrification rates. To compensate for that, and to provide an incubation environment that is similar to eutrophic reef communities, NO_3^- was added to the incubation water to preclude substrate limitation[29,44]. Both incubation water and headspace were 10 % acetylene enriched. Acetylene a) leads to a preferential reduction of acetylene to ethylene (hereafter C_2H_4) instead of N_2 to NH_4^+ by the nitrogenase enzyme[45], and b) inhibits denitrification at the nitrous oxide (N_2O) stage leading to an evolution of nitrous oxide[46]. Each chamber contained a single sample (scleractinian coral fragment, turf algae, or reef sediment core). Four or five respectively, replicate samples were incubated, additional two chambers without specimens served as controls to correct for planktonic background metabolism. During the 24 h incubations, chambers were submersed in a tempered water bath and stirred continuously (500 rpm) to ensure stable physical conditions and homogenous environment (27 °C, 12:12 h dark/light cycle, photon flux of ~200 µM quanta m^{-2} s^{-1}). Samples were taken at the beginning (t_0) and after 24 h (t_{24}). Both N_2O (for denitrification quantification) and C_2H_4 (for N_2 fixation quantification) concentrations were quantified by gas chromatography and helium pulsed discharge detector (Agilent 7890B GC system with HP–Plot/Q column, lower detection limits for both target gases were 0.3 ppm). Gas fluxes were corrected for the seawater control signal and normalised to the surface area of the organisms/substrates. C_2H_4 fluxes were converted into corresponding N_2 fluxes assuming a theoretical molar ratio of C_2H_4:N_2 = 4, which has been used in previous studies in similar environments[22,47].

Statistical analysis

The dataset was analysed using non–parametric permutational multivariate analysis of variance (PERMANOVA) using PRIMER–E version 6 software[48] with the PERMANOVA+ add on[49]. To test for differences in N_2 fixation, denitrification and O_2 fluxes between functional groups and eutrophied and control communities, 2–factorial PERMANOVAs were performed (factors were "functional group" and "control/eutrophied reef community"), based on Bray–Curtis similarities of square–root transformed data. Therefore, Type III (partial) sum of squares was used with unrestricted permutation of raw data (999 permutations), and PERMANOVA pairwise tests with parallel Monte Carlo tests were carried out when significant differences occurred. To identify correlations between N_2 fixation, denitrification, P_g, P_n and R, a Spearman–Rank–Order correlation was performed using SigmaPlot (SigmaPlot for Windows version 12.0).

8.4 | Results

Environmental parameters

Water temperature increased from 24.8 to 28.1 °C during the *in situ* manipulation period of eight weeks. Background DIN concentrations remained stable throughout the experiment at 0.40 ± 0.03. When nutrient manipulation was initiated, DIN concentrations increased up to seven–fold (measured directly at the communities) compared to background values (Figure 8.2). At the same time, PO_4^{3-} remained stable. For a detailed summary of nutrient concentrations at the centre of eutrophied communities, directly at the fertiliser pins and in surrounding waters, we refer to Supplementary Table S9.1.

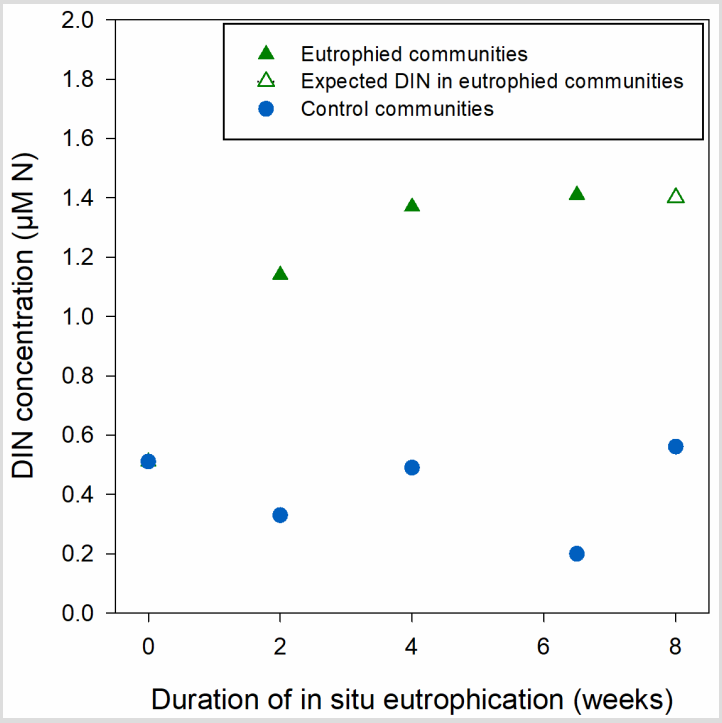

Figure 8.2 | Dissolved inorganic nitrogen (DIN) concentrations in control (blue) and eutrophied communities (green). Due to technical issues, no DIN concentrations were determined in eutrophied communities after eight weeks. Expected DIN concentrations, based on previous measurements are displayed here.

Oxygen fluxes

In both eutrophied and control communities (Figure 8.3), reef sediments showed lowest P_g being three to four times lower than in turf algae and five to six times lower than in *P. verrucosa*. A significant increase in P_g between control and eutrophied reef communities was only detected for reef sediments (Supplementary Table S8.2).

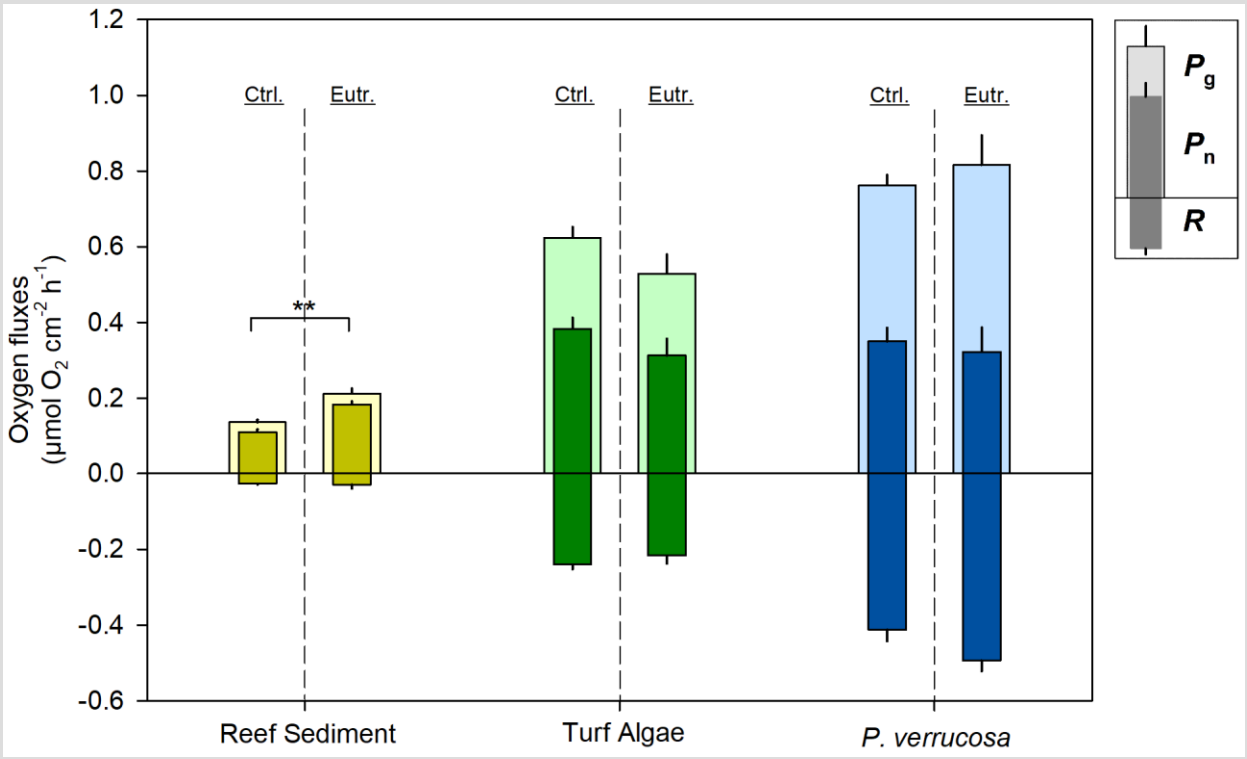

Figure 8.3 | Respiration (R), net photosynthesis (P_n) and gross photosynthesis (P_g) of three functional groups from control (Ctrl.; n = 5) and eutrophied (Eutr.; n = 4) communities. Asterisks indicate significant differences in P_g (** = p < 0.01). Data are presented in mean of replicates ± standard error of mean.

N_2 fixation and denitrification

Concentrations for both C_2H_4 and N_2O control incubations over the incubation time of 24 h were stable (Supplementary Figure S8.1). In control communities, turf algae showed the highest N_2 fixation activity (13.68 ± 1.42 nmol N_2 cm^{-2} d^{-1}) among the investigated functional groups, with N_2 fixation rates being 13 times higher than of sediments (Supplementary Table S8.3) and 274 times higher than the investigated scleractinian coral (Figure 8.4 and Supplementary Table S8.3). Denitrification rates did not differ significantly between investigated groups due to high variation, with *P. verrucosa* showing denitrification rates 2 times lower than turf algae and 2 to 3 times lower than reef sediments.

In eutrophied communities, N_2 fixation rates of turf algae (27.12 ± 1.57 nmol N_2 cm^{-2} d^{-1}) were 3 times higher than those of sediments (Supplementary Table S8.3) and 2 orders of magnitude higher than those of *P. verrucosa* (Supplementary Table S8.3). For sediments in manipulated reef communities, significantly higher N_2 fixation rates were observed compared to control communities (Supplementary Table S8.3), being 8–fold higher than without nutrient manipulation. Similar patterns of increasing N_2 fixation activity

were observed for turf algae and the scleractinian coral of eutrophic communities, though not statistically significant. In eutrophied communities, no statistical difference in denitrification rates between functional groups was observed. However, compared to untreated specimens, denitrification rates in functional groups from eutrophied reef communities increased significantly for turf algae (0.39 ± 0.03 nmol N_2O cm^{-2} d^{-1}; Supplementary Table S8.2). Denitrification rates increased, though not significantly (Supplementary Table S8.2), 3–fold in sediments (0.49 ± 0.08 nmol N_2O cm^{-2} d^{-1}) and 5–fold for *P. verrucosa* (0.20 ± 0.06 nmol N_2O cm^{-2} d^{-1}) compared to control communities.

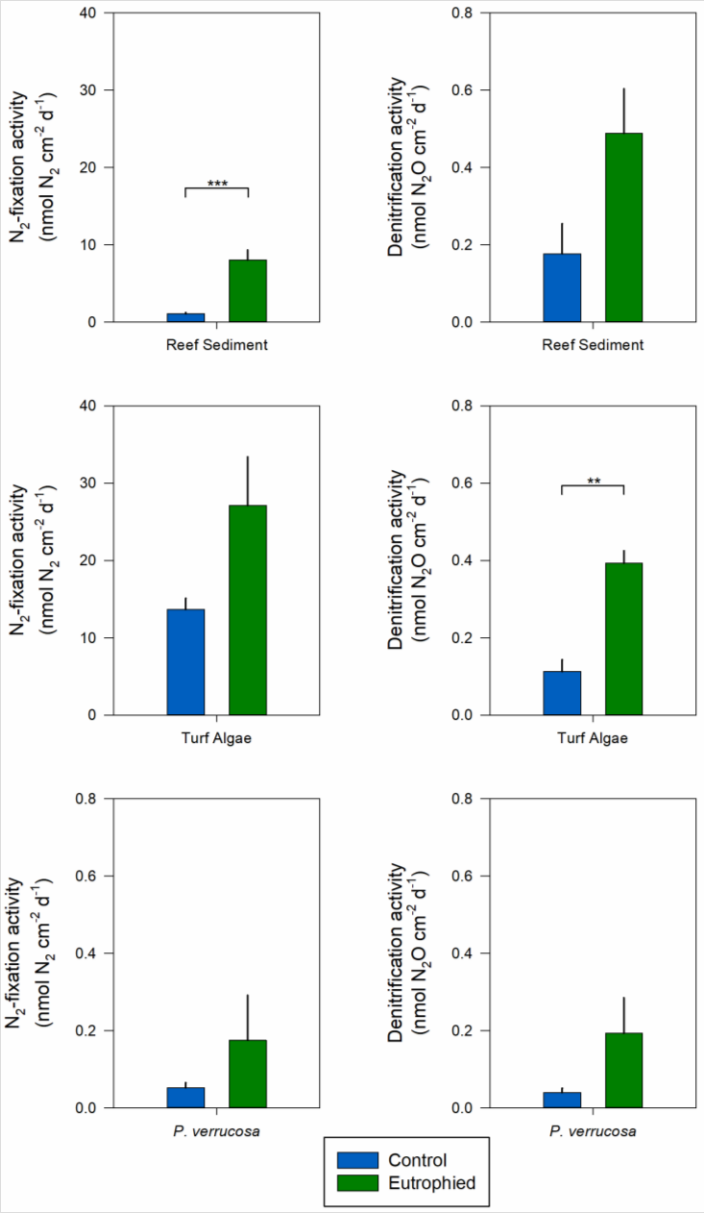

Figure 8.4 | N_2 fixation (left figures) and denitrification (right figures) activity of investigated functional groups from control (blue bars; n = 5) and eutrophied (green; n = 4) communities. Asterisks indicate significant differences (** = $p < 0.01$, *** = $p < 0.001$). Data are presented in mean of replicates ± standard error of mean. Note different scale for denitrification and N_2 fixation and for N_2 fixation in *Pocillopora verrucosa*.

We observed positive correlations between N_2 fixation and denitrification (r_s = 0.480, p = 0.012; Table 8.1), and between N_2 fixation and R (r_s = 0.454, p = 0.018; Table 8.1), and P_n and P_g (r_s = 0.843, p < 0.001; Table 8.1). Negative correlations were identified for R and P_n (r_s = –0.621, p < 0.001; Table 8.1), along with R and P_g (r_s = –0.891, p < 0.001; Table 8.1).

Table 8.1 | Spearman-Rank-Order correlation coefficients (r_s) between N_2 fixation, denitrification, respiration (R), net primary production (P_n) and gross primary production (P_g).

	Denitrification	R	P_n	P_g
N_2 fixation	0.480*	0.454*	0.116	-0.311
Denitrification		0.332	-0.221	-0.256
R			-0.621***	-0.891***
P_n				0.843***

Pairs of variables with positive correlation coefficients and significant p-values < 0.05 tend to increase together, for pairs with negative correlation coefficient and p-values < 0.05, one variable tends to decrease while the other increases. * p < 0.05. *** p < 0.001.

8.5 | Discussion

Anthropogenically induced nutrient inputs to coral reefs have multi–level impacts[12,50]. This study extends previous work of Koop et al.[14] and Capone et al.[51] by showing that increasing DIN concentrations alter essential biochemical processes such as primary productivity, N_2 fixation and denitrification in coral reef communities.

Environmental parameters

As background DIN concentrations remained stable throughout the experiment (Figure 8.2), we are confident that further pulses of nutrient inputs, e.g., through terrestrial runoffs, were not present. DIN concentrations in experimentally nutrient–enriched communities constantly exceeded concentrations of control communities and were up to 7–fold higher. Compared to other studies, in which manipulative eutrophication was performed[11,14], DIN concentrations in eutrophied communities of the current experiments were about 4 to 39 times lower and reflect a more realistic ecological scenario in the context of the oligotrophic Red Sea[52,53]. We here simulated eutrophication in a season in which DIN concentrations are usually low (compared to Roth et al.[36]). The manipulation can, thus, be considered as an unnaturally appearing moderate eutrophication event simulating nutrient inputs in the Red Sea from point–sources such as aquaculture[16] or urban wastewater[28]. Still, eutrophied communities of the present study experienced DIN concentrations that were higher than the "eutrophication thresholds" of ~1.0 µM suggested by Lapointe[3], which was also referred to in a previous study in the Red Sea[54], confirming a successful enrichment over the eight–week manipulation period.

N_2 fixation and denitrification under elevated nutrients

Results of the study showed increasing N_2 fixation and denitrification in all functional groups in response to higher nutrient availability compared to control communities. High variability caused a lack of statistical significances which could be explained by low replication (n = 4 or n = 5, resp.). Descriptive trends for increasing N_2 fixation and denitrification were homogenous for all functional groups of eutrophied communities, and are referred to hereafter when discussed. Potentially, high variability could be

counteracted by higher replication. For N_2 fixation, these findings contradict our expectations and observations from previous studies that found reduced N_2 fixation activities under high DIN availability in coral reef–associated sediments[14,32]. For denitrification, this is in line with other studies that have demonstrated increased denitrification activities in coral reef–associated sediments under elevated nutrient availability[14,51].

Theoretically, an energetically more cost–efficient alternative to N_2 fixation is the assimilation of N (in the form of NO_3^- and/or NH_4^+), which was provided by performed *in situ* manipulation[32,55]. As a result, we expected a lower N_2 fixation activity. However, a stimulating effect on N_2 fixation in response to increased DIN in surrounding waters was observed. Generally, the activity of diazotrophs is inhibited by elevated N availability, as shown in mesotrophic or eutrophic lakes[56], sediments in bays[29], seagrass roots in an estuary[57] and in agricultural crops[58]. However, the role of nutrients on N_2 fixation remains arguable, as there is also continued N_2 fixation activity in response to elevated DIN concentrations of up to 30 µM NO_3[32]. Even stimulating effects by providing N have been observed[59], likely explained by the added form of combined N (Chloramphenicol) and the time of day when respective incubations were initiated (afternoon).

The converse response of N_2 fixation to elevated N concentrations reported by many studies highlights that N–cycling processes are impacted by multiple environmental factors. For example, in general, both N–cycling processes are performed in anaerobic milieus: elevated O_2 concentrations can result in a depression of nitrogenase activity and subsequently in lower N_2 fixation activity[60]; likewise, denitrification is mediated by anaerobic bacteria[61] and thus also depends (besides other factors) on low O_2 availability[62]. Therefore, we tested whether O_2 concentrations explained the changes in both N–cycling pathways[63]. However, the lack of a correlation between N–cycling pathways and P_n and/or P_g excluded O_2 as a potential driver for both N_2 fixation and denitrification activity. We, thus, hypothesise that both processes may be spatially or temporally separated from O_2 evolution in reef sediments, turf algae and *Pocillopora verrucosa*[64]. A temporal separation in hard corals can occur due to heavily varying O_2 concentrations within boundary layers, ranging from super–saturation during daylight to anoxia at night, caused by metabolic processes of the coral host and Symbiodiniaceae[65]. Especially these alternating changes from anaerobic to aerobic conditions can even fuel denitrification, as nitrification, i.e. the oxidation from NH_4^+ to NO_2^- and NO_3^-, is stimulated by the presence of O_2[66], subsequently resulting in the formation of NO_3^- which serves as a substrate for denitrification[44]. Alternatively, increased N_2 fixation and denitrification in all groups indicate that the involved N–cycling prokaryotes are capable of performing N_2 fixation and denitrification in the presence of O_2[24]. From this, we conclude that N_2 fixation and denitrification activities were not directly altered by the presence of O_2.

Besides an oxic–anoxic environment, the availability of organic C (hereafter C_{org}) can be decisive for diazotrophs and denitrifiers, as it poses as an important energy source[67,68]. C_{org} can be acquired via the uptake from the water column[69], or, in coral holobionts, it is translocated by C–rich photosynthates from symbionts[25].

We here report elevated P_g activity in reef sediments of eutrophied communities compared to the control, suggesting increased C_{org} in reef sediments, which was potentially caused by higher photosynthetic rates of epilithic algae on the sediment as described by Cook et al.[70]. Additionally, the export of C_{org} from neighbouring turf algal assemblages in the form of dissolved organic carbon (hereafter DOC)[71], with a subsequent DOC uptake by reef sediments[72], potentially provides sufficient C_{org} as an energy source

leading to increasing N_2 fixation activities. We thus conclude, that even though N of the fertiliser pins was not taken up by reef sediments directly[73], an indirect effect via the interplay with other functional groups lead to a stimulation of N_2 fixation. This, along with findings of a related study[73] indicates that reef sediments and their associated microbial community were not N–limited.

For turf algae, denitrification rates measured in eutrophied communities were significantly higher than those from control communities. Similarly, N_2 fixation was increased in turf algae from eutrophied communities. Our data reveal no changes in P_g, P_n and R in turf algae from eutrophied communities compared to controls; thus, no further (direct) source of energy was provided for N cycling processes. Turf algae can, however, be highly flexible in acquiring N from different sources. For example, N_2 fixation in turf algae was highest among all measured functional groups, thus, this process contributed considerably to satisfy N demands in control communities[47,74]. Shifts to a preferable uptake of allochthonous N in eutrophied communities[73] were detected, emphasising the turf algae's flexibility, and underlining their role as opportunists efficiently taking up environmentally offered N[27]. We support the idea of turf algae assemblages being N–limited and benefiting from increased DIN concentrations. In this context, the ability to take up N fast[27], as well as assimilate and process N compounds can subsequently result in rapid takeover of bare substrates[35,36], which underlines the competitiveness of turf algae under elevated nutrient concentrations[75]. Thus, an assumed incorporation and processing of N can ultimately enhance microbial growth as assimilates can be stored or used for metabolic processes[76]. We suggest a conterminously increasing abundance of diazotrophs[77] and likely denitrifiers, which could explain the increased N_2 fixation and denitrification activities, although microbial communities may vary and respond strongly to environmental changes.

In *P. verrucosa*, we observed increasing N_2 fixation and denitrification rates in eutrophied communities compared to controls. Stimulated N_2 fixation by elevated DIN concentrations contradicts expected patterns. Potentially, longer experiments (36 months)[12] and/or longer experiments mimicking a more severe eutrophication event (12 months, 36.2 µM NH_4^+)[14] could lead to suppressed N_2 fixation rates in coral holobionts. This underlines that elevated nutrient concentrations not necessarily negatively impact coral holobionts under certain conditions. Indeed, Atkinson et al.[78] demonstrated that hard corals can flourish across a wide range of nutrients without showing signs of stress. Moreover, Bongiorni et al.[79] showed that increased nutrient availability can promote coral growth. Thus, we speculate that nutrient enrichment (i.e., eutrophication) may not always negatively impact scleractinian corals[80,81]. Additionally, it has been demonstrated that *P. verrucosa* harbours a stable, rather inflexible microbial composition[82]. This ultimately questions whether the N_2 fixation pathway is continually facultative as previously assumed[25], or if it can be considered obligate to some extent under certain conditions, a N_2 fixation strategy that has been observed in terrestrial ecosystems[83]. Moreover, stimulated denitrification and N_2 fixation activity through increased DIN concentrations suggest that both denitrification and N_2 fixation may potentially be carried out – at least to a certain degree – by the same microbes, as previously postulated for seagrass associated bacteria[84,85]. Furthermore, we assume a shift from originally N[3,6,86] towards P limitation[11] and/or other micronutrients[20,50] in *P. verrucosa* from treated communities. This is likely occurring when excess inorganic N is available[11]. We here utilised fertiliser bags for manipulation that also contained P. Although P concentrations were increased at the fertiliser pins directly (Supplementary Table S9.1), the role of P in our study remains speculative, as no increase inside the eutrophied communities was observed. This can be explained either by a) an immediate uptake by benthic and pelagic organisms[87,88] as P is considered

crucial[89] and limiting[86,90] for primary productivity; or b) a discontinuous P supply from the fertiliser due to solubility and quick leaching. By all means, N_2 fixation is often limited by micronutrients[20], so that even minor changes in their availability may result in N_2 fixation stimulation, as reported for many marine and limnetic systems[91]. The assessment of P enrichment effects on coral reefs by D'Angelo & Wiedenmann[50] supports this hypothesis, as they describe the complex dimension of a rapid P utilisation by N–fixing *Trichodesmium* transforming high P levels into P–depleted conditions.

Ecological implications

This the first study showing that microbial N cycling in tropical coral reef communities may not provide an effective relief and may even exacerbate anthropogenic eutrophication due to stimulated N_2 fixation. We further posit that N_2–fixers are rather inflexible to respond to anthropogenic N inputs (i.e., increasing N inputs to coastal ecosystems)[3] under certain conditions. Extrapolating these findings in the light of climate change, we suggest that microbial N cycling may contribute to N oversupply and thereby increases the likelihood of phase shifts from a coral–dominated to an algae–dominated reef[4].

Released nutrients from the fertiliser pins added on a low baseline, even though final DIN concentrations did not exceed natural occurring DIN fluctuations[36]. Thus, the observed N_2 fixation and denitrification stimulation in the present study conceivably occur due to the inflexibility to respond to anthropogenically induced DIN changes, even though the capacity to react to natural DIN inconstancies has been demonstrated before[22,47]. Additionally, we suspect that specific DIN thresholds, which regulate the activity of N_2 fixation and subsequently also of denitrification, have not been exceeded in the present study.

Moreover, denitrification is only one mechanism within the N cycle that can relieve coral reef ecosystems from excessive N. Further processes, such as anaerobic ammonium oxidation (ANAMMOX) transform fixed ammonium into elemental N_2, thereby removing bioavailable N from the system. ANAMMOX occurs in many marine environments[92,93] and has been detected in coral reef–associated sponges[94]. Likely, it also appears in coral holobionts[25], for which the role of ANAMMOX in coral reefs needs to be targeted in future studies.

Synoptically, future research should, thus, aim to a) address the effects of severe eutrophication on N_2 fixation and denitrification in coral reef communities and by that identifying DIN thresholds by which N_2 fixation is suppressed; b) detect and quantify the interaction between N_2 fixation and denitrification with other N cycling pathways (i.e., nitrification and ANAMMOX) and c) identify a coral reef community–wide N_2 fixation and denitrification budget under ambient and stressed scenarios, precisely because the role of symbiotic and planktonic diazotrophs providing fixed N in bleached corals is still under debate[90,95].

8.6 | Acknowledgments

This work was funded by the German Research Association (DFG Project: Wi 2677/9–1) to CW and KAUST baseline funding to BHJ and CRV. We are thankful to Rüdiger Willhaus for his expertise in graph visualisation, and to Najeh Kharbatia for technical support.

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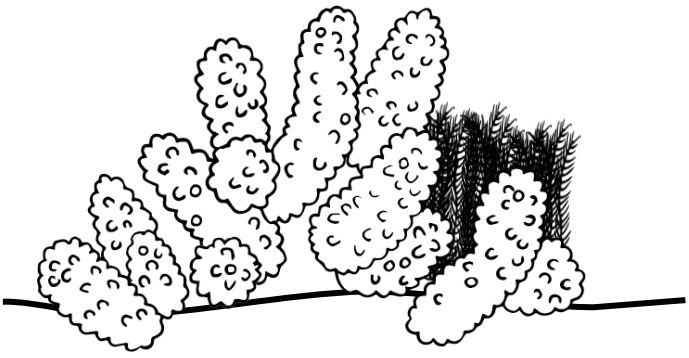
Chapter 9



Chapter 9 | **Nitrogen eutrophication particularly promotes turf algae in coral reefs of the central Red Sea**

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9.1 | Abstract

While various sources increasingly release nutrients to the Red Sea, knowledge about their effects on benthic coral reef communities is scarce. Here, we provide the first comparative assessment of the response of all major benthic groups (hard and soft corals, turf algae and reef sands—together accounting for 80 % of the benthic reef community) to in−situ eutrophication in a central Red Sea coral reef. For 8 weeks, dissolved inorganic nitrogen (DIN) concentrations were experimentally increased 3−fold above environmental background concentrations around natural benthic reef communities using a slow release fertilizer with 15 % total nitrogen (N) content. We investigated which major functional groups took up the available N, and how this changed organic carbon (C_{org}) and N contents using elemental and stable isotope measurements. Findings revealed that hard corals (in their tissue), soft corals and turf algae incorporated fertilizer N as indicated by significant increases in $\delta^{15}N$ by 8 %, 27 % and 28 %, respectively. Among the investigated groups, C_{org} content significantly increased in sediments (+24 %) and in turf algae (+33 %). Altogether, this suggests that among the benthic organisms only turf algae were limited by N availability and thus benefited most from N addition. Thereby, based on higher C_{org} content, turf algae potentially gained competitive advantage over, for example, hard corals. Local management should, thus, particularly address DIN eutrophication by coastal development and consider the role of turf algae as potential bioindicator for eutrophication.

Keywords: Coral reefs | nutrients | stable isotopes | nitrogen cycling | eutrophication | turf algae | zooxanthellae | phase shifts

A modified version of this chapter has been published in *PeerJ* 8:e8737
https://doi.org/10.7717/peerj.8737

9.2 | Introduction

Coral reefs are among the most productive and biologically diverse ecosystems on the planet[1], even though they grow in oligotrophic waters of the tropics[2]. The young and isolated Red Sea, with its thriving coral reefs, is highly oligotrophic, particularly in the subtropical central and northern areas[3–6]. However, nutrient inputs to the Red Sea from aquaculture[7–10] and urban waste water[5,11–13] affect marine life[7,11,14,15]. At the same time, the expansion of aquaculture industries in view of the Saudi Arabian coastal development agenda (https://vision2030.gov.sa/en/node), and growing urban sources, for example, from the city of Jeddah with about 4.6 Mio. inhabitants[16], represent further stressors to coral reefs in the Red Sea. Significant parts of the city rely on septic tanks for wastewater which can be a source of nutrients and pollutants through leakages into the groundwater[17,18]. Moreover, the discharge of insufficiently treated sewage from marine outfalls (i.e., pipe discharge) as a point−source[12,19] was already shown to raise near−shore N availability[4], affect planktonic[20] and coral[21] microbial communities and reach nearby reefs[13,19]. As nutrients, among several stressors, have the largest effect on Red Sea hard coral resilience to climate change[22], a deeper understanding of the community response to eutrophication is fundamental.

Benthic coral reef communities are crucial for many ecosystem functions, including the cycling and retention of carbon (C) and nitrogen (N)[23–25] but suffer from anthropogenic disturbances[26–28]. N availability is an important limiting factor for the biological productivity in oligotrophic reef environments[29]. Local eutrophication may impact reef organisms that typically grow in nutrient−poor waters[15], and the diverse array of metabolisms they are comprised of. One prominent example is the entirety of coral host, endosymbiotic algae (zooxanthellae), bacteria and other microorganisms[30], called the coral holobiont. The enrichment source[31,32] and ratio of supplied nutrients is important to determine reef biota's reactions to eutrophication, particularly for corals[33,34]. Metabolic differences, for example between autotrophic and heterotrophic lifestyles, as well as the feeding environment of heterotrophic organisms, can lead to imbalances of essential biochemicals, which may become limiting[35]. Critical parameters to evaluate and trace nutrient fluxes as well as limitations in marine environments are the C and N elemental[36–40] and isotopic[5,41,42] composition. N uptake and circulation in the reef might be fast and while the input of N can be measured by the long−term increase in forms of N concentrations[43], it is most directly traceable in the short−term by the isotopic signature of reef biota. As external sources and processes of N acquisition affect the isotopic composition, for example, of corals[44], anthropogenic N sources can be traced in the field[42,45,46]. N enrichment has negative effects on coral growth[22,47,48], calcification[49,50], reproductive success[7,48,51], biodiversity[52], bacterial communities[22] and increases the susceptibility of corals to bleaching[32,34,53,54]. In contrast, other benthic groups in coral reefs, such as turf− and macroalgae benefit from increased nutrient availability in many cases[55,56], particularly in combination with reduced herbivory. Hence, shifts from coral− to algal−dominated reefs, so−called phase shifts, can occur[57,58].

While extensive research investigated the causes of phase shifts[59,60], nutrient effects on the ecophysiology and elemental stoichiometry of reef functional groups are rarely assessed, overlooking connections between uptake to utilization. Responding to the growing nutrient inputs to the central Red Sea, an assessment of their effects on coral reef communities is needed in this originally nutrient poor region, particularly which functional groups and ecophysiological parameters may indicate early−stage effects. Reefs in the oligotrophic Red Sea can serve as a "natural laboratory"[61–63], as anthropogenic nutrient inputs add on a comparably low baseline. However, most studies have been conducted in the laboratory rather than in−situ, with associated risks of experimental artifacts, oversimplification or overestimation[64]. Indeed,

local boundary layers and contact zones are of major importance in terms of direct interaction, small scale flow regimes as well as accumulation and transfer of organic matter[65,66], which can hardly be simulated under controlled laboratory conditions. The few similar studies that exist were conducted in less oligotrophic seas[48,67], along the natural environmental gradient of the Red Sea[5], focused on one individual benthic group only[7,39,68], or only investigated benthic cover or chlorophyl content, not considering other metabolic parameters[15,33].

Therefore, we assessed the responses of major benthic functional groups (hard corals (*Pocillopora* cf. *verrucosa*, that is, tissue and zooxanthellae), soft corals (Xeniidae), turf algae and sediments) to N enrichment through a manipulative in−situ experiment in the central Red Sea. Combining elemental and stable isotope analysis, this approach provides information starting from N in the water column, through N uptake, to its utilization. We address the following underlying research questions: (1) Which major functional groups take up available N and (2) how did this affect organic carbon (C_{org}) and N contents? Taken together, we aimed to draw conclusions about nutrient limitation for different functional groups.

9.3 | Materials and Methods

Study site and environmental conditions

The experiments were conducted at Abu Shoosha reef (22°18’15”N, 39°02’56”E) on the west coast of Saudi Arabia in the central Red Sea from late January until late March 2018. The reef assessed in this study does not fall under any legislative protection or special designation as a protected area. Under the auspices of KAUST (King Abdullah University of Science and Technology, Thuwal, Saudi Arabia), sailing permits to the reef were granted that included the collection of corals and other reef benthos. This reef is characterized by generally high levels of herbivory and small fluctuations in ambient dissolved inorganic nitrogen (DIN) concentration during this period[6]. For example, in January to March of the previous year (i.e., 2017), sea water concentrations of ammonium (NH_4^+) ranged from 0.16 to 0.17 µM, nitrate (NO_3^-) from 0.25 to 0.40 µM, nitrite (NO_2^-) from 0.03 to 0.06 µM, phosphate (PO_4^{3-}) from 0.02 to 0.21 µM and the resulting DIN(NO_3^- + NO_2 + NH_4^+)/PO_4^{3-} ratio from 2.9:1 to 20:1[6]. Abu Shosha reef features turf algae (37 %) and hard corals (29 %) as most abundant functional groups (Table 9.1).

Table 9.1 | Relative benthic cover of functional groups at the experimental reef.

Major functional groups	Cover (%)
Filamentous turf algae	36.8
Hard coral	28.8
Rubble	10.2
Biogenic rock	8.7
Soft coral	8.5
Sediment	6.0
Tridacna sp.	0.7
Macroalgae	0.4

Data taken from Roth et al.[6]

Key environmental variables were monitored every 2–3 weeks at the sampling site, as described in a related study by Roth et al.[6]. Briefly, water temperature was measured with continuous data loggers (Onset HOBO Water Temperature Pro v2 Data Logger—U22−001; accuracy: ±0.21 °C) and are given in

3−day means (72 h). For background measurements of dissolved NO_3^-, NO_2^- and PO_4^{3-}, water samples were taken in triplicates at the study site at least 2 m away from any fertilizer source (see "Experimental Design and Sampling Strategy" for more details). Water samples were filtered on the boat (Isopore™ membrane filters, 0.2 µm GTTP) and stored dark and cool until they were frozen to −50 °C in the lab. Nutrient concentrations were determined with a continuous flow analyzer (AA3 HR, SEAL). The limits of quantification (LOQ) for NO_3^-, NO_2^- and PO_4^{3-} were 0.084 µmol L^{-1}, 0.011 µmol L^{-1} and 0.043 µmol L^{-1} respectively. Five mL subsamples for NH_4^+ were filtered into separate acid washed centrifuge tubes. A total of 1.2 mL ortho−phthalaldidehydesolution (OPA) was added, and samples were incubated > 4 h with OPA in the dark. NH_4^+ concentrations were determined fluorometrically within 8 h (Trilogy® Laboratory Fluorometer; Turner Designs Inc., San Jose, CA, USA). The LOQ for NH_4^+ was 0.094 µmol L^{-1}. The sum of NO_3^-, NO_2^- and NH_4^+ concentrations reflect DIN.

Experimental design and sampling strategy

Eight distinct patches of reef communities, each surrounded by patches of reef sand, were chosen in the back reef of Abu Shoosha at a water depth of approximately 5 m. The chosen communities, which represented the surrounding reef in composition (Table 9.1), were exposed to simulated eutrophication for 8 weeks in total. More specifically, each of the replicate communities was surrounded by four pins with approximately 70 g of slow release fertilizer granulate (Osmocote® Plus (15−9−12)) (Figure 9.1). Being one of the most commonly used fertilizers for eutrophication experiments[40,69–71], this approach provides a fast and high supply of macronutrients (15 % total N (8 % nitrate N, 7 % ammoniacal N), 9 % available phosphate, 12 % soluble potash) from the 1st day of fertilization under local temperature regimes[72]. Osmocote® Plus (15−9−12) provides a balanced fertilization of N and phosphorus (P), however, only the fate of N was considered in this experiment as particularly N effects were of interest. The fertilizer was renewed every 2–3 weeks to assure a continuous nutrient supply[72]. To test whether the nutrient addition was effective locally, water samples for nutrients were taken directly at the fertilizer pin, and 25 cm towards the manipulated communities according to the protocol outlined above.

The effect of eutrophication was then assessed at the major functional groups (in terms of benthic reef cover in the central Red Sea) that were present in the selected communities. Specifically, we chose autotrophic hard corals (*Pocillopora* cf. *verrucosa)*, soft corals (Xeniidae), turf algae and reef sand (sediments). These groups covered ∼80 % of the sampled reef[6]. Turf algae were defined as dense and flat (less than 2 cm in height) assemblages of filamentous algae of different species, including small individuals of macroalgae and cyanobacteria.

Manipulated specimens ("treatment") were sampled from within a close radius (∼25 cm) of the fertilizer tubes. As the in−situ communities were also needed in other experiments investigating their C chemistry (Roth et al., in review) and N fluxes (El-Khaled et al., Chapter 8 of this thesis), specimens for natural conditions at the beginning ("start") of the experiment were collected in the surrounding reef. Additional samples in replicates of eight were collected in the surrounding non−fertilized reef at the end ("control") of the experiment, to reflect non−fertilized control conditions. For start and control data, a distance of at least 10 m from any fertilizer pin was maintained and the same depth as well as light conditions were given.

Samples were acquired with hammer and chisel. Hard coral and turf fragments (their substrate) were of approx. 10 cm in length. Sediments were collected using a Petri dish, which was dragged into the sediment

upside−down (max. depth 14 mm) and the sediment was fixed to the dish from underneath. The samples were stored at −80 °C until further preparation. Hard corals, soft corals and turf algae were rinsed with Milli−Q to remove excess salt. Epilithic turf algae were scraped off from their surface with a scalpel and tweezers.

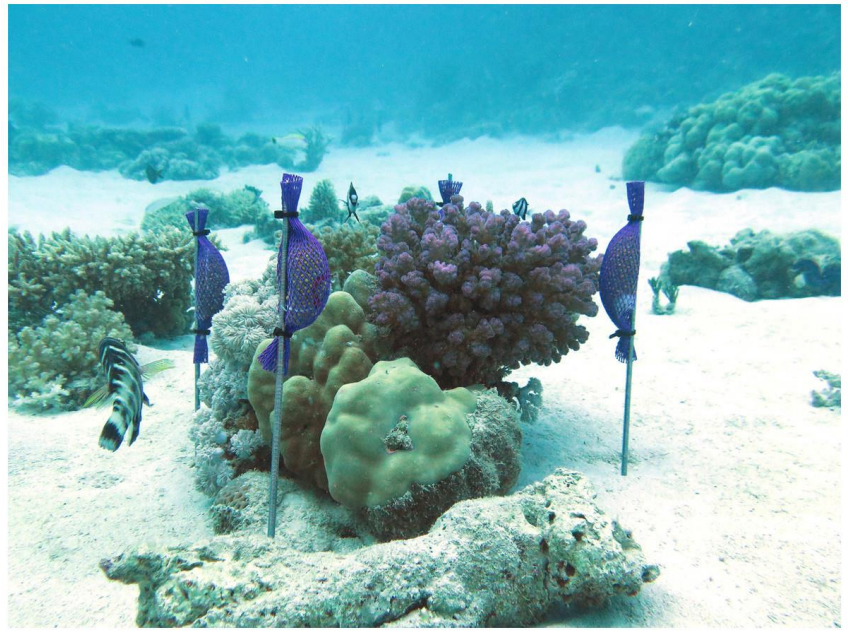

Figure 9.1 | Example of a manipulated in-situ community surrounded by four pins with attached fertilizer (Osmocote®) bags, photo: Florian Roth.

Elemental and stable isotopic compositions of C and N

Turf algae, soft corals and sediments were dried for 48 h (sediments: 72 h) at 40 °C. Following Jessen et al.[39], hard coral tissue was removed using an airbrush, and the resulting tissue slurry was weighed, homogenized (MicroDisTec 125) and centrifuged for 10 min (Eppendorf Centrifuge 5,430 R, 4 °C, 3,220 rcf) to separate algae ("zooxanthellae") from animal "tissue". The supernatant was filtered (Whatman, GF/F) and for each sample two filters were generated. Filters for N and inorganic C analysis were dried for 24 h at 40 °C. Filters for C_{org} measurements were exposed to HCl fumes (from 37 % HCl) and dried for 24 h. The remaining zooxanthellae pellet was dried for 48 h at 40 °C.

Sub−samples of all groups were ground using an agate mortar and pestle. A mill grinder (Retsch, PM 200, 4 min) was used for the sediments. For preparation of C_{org} samples, 5–10 g of ground sediment were placed in an Erlenmeyer flask and covered with Milli−Q. Drops of HCl (37 %) were added until the reaction ceased. The acidified liquid was transferred to 50 mL Falcon tubes which were filled up with Milli−Q, to stepwise wash the sample pellet and raise the pH up to neutrality, and subsequently centrifuged for 10 min (Eppendorf Centrifuge 5,430 R, 4 °C, 7,200 rcf). The liquid supernatant was discarded and tubes were then refilled with Milli−Q for 3–4 times to raise pH. The pellets were dried in the Falcon tubes for 48 h at 40 °C. The dry, homogenous powder was analyzed for: (a) N and inorganic C quantities; and (b) C_{org} as in Roth et al.[6]. C_{org}/N ratios, fractions of organic and inorganic C and isotope ratios were measured as in Rix et

al.[73] using a Flash 1112 EA coupled to a Delta V IRMS via a ConfloIV−interface (Thermo Scientific, Waltham, MA, USA). Isotopic ratios are shown as $\delta^{13}C$ or $\delta^{15}N$ (‰) = ($R_{sample}/R_{ref} - 1$) × 1,000. There, R is the ratio of heavier:lighter isotope (^{13}C:^{12}C or ^{15}N:^{14}N). As reference, Vienna Pee Dee Belemnite was used for C (R_{ref} = 0.01118) and atmospheric nitrogen for N (R_{ref} = 0.00368).

Zooxanthellae cell density and mitotic index

For hard corals, zooxanthellae cell density and the mitotic index were analyzed following the described sampling strategy (start, control and treatment), whereby "start" and "control" were from the surrounding reef. Aliquots of 20 µL homogenized tissue sample and 80 µL Milli−Q were vortexed (Gilson, GVLab) directly before taking 10 µL on an improved Neubauer Levy hemocytometer (0.0100 mm deep). Pictures were taken with a ZEISS Primovert microscope via Labscope (Version 2.5) from the 5 × 5 grid in 40−fold and randomly in 20−fold magnification. Manual counts of zooxanthellae and the mitotic index were related to the total amount of airbrushed slurry per individual. Here, clumps and inhomogeneous patches were not considered. For normalization to the coral surface area, 3D models for all coral skeletons were generated using the software AutodeskReCap Photo (v18.2.0.8).

Data analysis

Nitrogen uptake by functional groups was assessed using stable isotope analysis. N utilization was assessed by elemental analysis, and C_{org}/N ratios served to identify nutrient limitations[37,38,74,75], along with zooxanthellae cell density and mitotic index (for hard corals). Statistical analysis was conducted with RStudio[76]. Xeniidae and *Pocillopora* cf. *verrucosa* were not abundant in all eight communities (only in 5 and 6, respectively). Due to logistical constraints, "start" data of soft corals was not available. A two−way ANOVA (factors: treatment, dominant functional group) showed no significant effect of community composition on our response parameters under N eutrophication, therefore data from more coral and more algae dominated communities were pooled. Significant differences between "start" and "control" as well as between "control" and "treatment" were checked with two−sample *t*−tests (test statistic: *t*) if test assumptions were fulfilled, otherwise Mann–Whitney–Wilcoxon Tests (test statistic: *W*) were applied. A similar approach was conducted for cell density of zooxanthellae and mitotic index per treatment. Tissue homogenization of the "start" samples was visually much worse than for "treatment" and "control" samples, but is shown for completeness and homogeneity.

9.4 | Results

Environmental parameters and N availability

During the study period, water temperature increased from 25 °C to 28 °C (Supplementary Table S9.1). The mean background concentration in sea water for DIN was 0.34 ± 0.07 µM N and 0.10 ± 0.02 µM PO_4^{3-} during the time of the experiment (measured after 2, 4 and 6.5 weeks). Accordingly, the environmental background DIN/PO_4^{3-} ratio was 3.4 (±0.08):1 on average. The manipulation of nutrients increased DIN concentrations (measured after 2, 4 and 6.5 weeks) on average 3−fold and to a maximum of 7−fold directly at the communities, relative to background concentrations (Figure 9.2). Namely, manipulated NO_3^- was 1.05 ± 0.09 µM and manipulated NH_4^+ was 0.22 ± 0.06 µM. PO_4^{3-} remained at ambient condition, despite being present in the fertilizer (Supplementary Table S9.1). As such, the mean DIN/PO_4^{3-} ratio at the manipulated communities was 15.1 (±3.46):1.

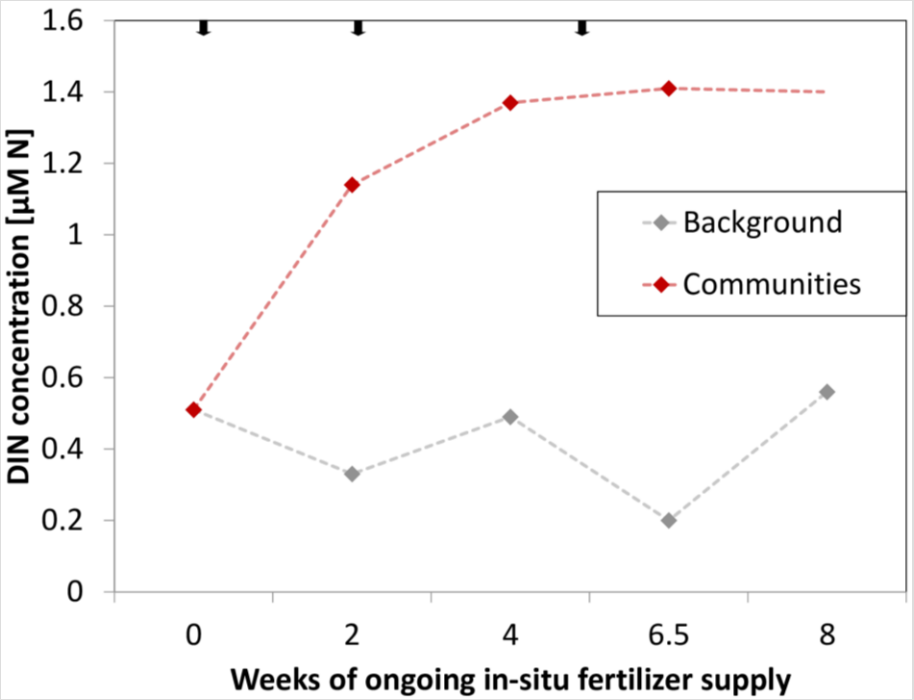

Figure 9.2 | Dissolved inorganic nitrogen (DIN) concentrations at experimental communities (red, last data point extrapolated) and of background sea water (grey) over time of in-situ manipulation. Black arrows symbolize introduction and renewal of fertilizer.

Uptake of excess N by benthic functional groups

Pure Osmocote® fertilizer was enriched in ^{15}N ($\delta^{15}N$ = 16.326 ± 0.257) (Supplementary Table S9.2). Hard corals (tissue), turf algae and soft corals took up excess N, as indicated by significantly (t_{12} = 2.553, p = 0.025; t_{13} = 3.228, p = 0.007; t_9 = 6.705, p < 0.001, respectively) increased $\delta^{15}N$ (Figure 9.3A). The $\delta^{15}N$ values in manipulated functional groups were 8 % (*Pocillopora* tissue), 27 % (Xeniidae) and 28 % (turf algae) higher compared to untreated controls after the same time.

Utilization of excess N by benthic functional groups

Nitrogen content was highest in hard coral zooxanthellae both before and after eutrophication (Figure 9.3B). In the eutrophication treatment, N content was significantly higher in the tissues of Xeniidae (t_9 = 5.667, p < 0.001) and turf algae (W = 49, p = 0.014). Their tissues contained 85 % (Xeniidae) and 39 % (turf) more N compared to untreated controls. Increases in the other groups were not significant.

To investigate whether N was utilized to produce C_{org} under a metabolically stable ratio, the C_{org} content and C_{org}/N ratio are presented. The hard coral components showed the highest C_{org} content ranging from 50.39 ± 1.83 % to 55.05 ± 1.49 % in the tissue and between 42.64 ± 1.39 % and 45.78 ± 1.49 % in zooxanthellae (Figure 9.3C). Minimum C_{org} content was observed in reef sediments, ranging between 0.09 ± 0.01 % and 0.11 ± 0.01 %. Only turf algae and sediments showed a significant change in C_{org} content (t_{14} = 2.568, p = 0.022; t_{14} = 2.537, p = 0.023, respectively). This represents an increase in C_{org} content by 33 % in turf algae and 24 % in sediments in the treatment compared control specimen.

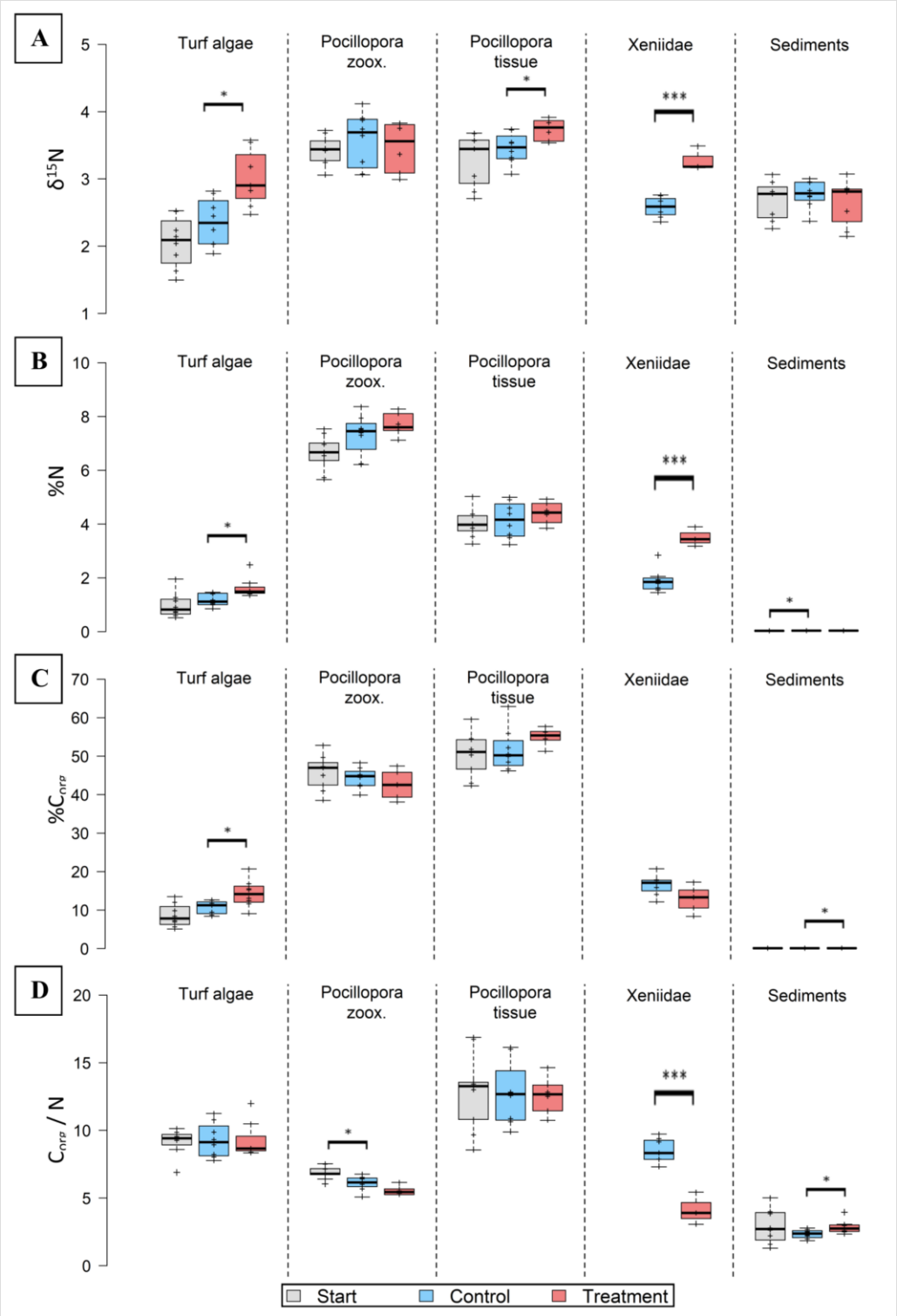

Figure 9.3 | Nitrogen (N) and carbon elemental and N isotopic composition of major functional groups before (grey), without (blue) and after 8 weeks in-situ eutrophication (red). Investigated groups are turf algae, *Pocillopora* cf. *verrucosa* zooxanthellae ("zoox.") and -tissue, Xeniidae and sediments. Eight replicates per boxplot. **(A)** Nitrogen isotopes ($\delta^{15}N$), **(B)** nitrogen content (%N), **(C)** organic carbon content ($\%C_{org}$), **(D)** organic carbon to nitrogen ratio (C_{org}/N). Asterisks indicate significant differences (*$p < 0.05$ and **$p < 0.001$).

The C_{org}/N ratio for treated Xeniidae was significantly lower (t_8 = −6.405, p < 0.001) than for Xeniidae in the surrounding reef (8.520 ± 0.320 compared to 4.132 ± 0.566) (Figure 9.3D). In sediments of the eutrophication "treatment" the C_{org}/N ratio was significantly higher than in controls (W = 53, p = 0.028), however it did not increase compared to start values. In hard coral zooxanthellae, C_{org}/N ratio declined over time but was not significantly different in treatment data compared to controls. C_{org}/N remained constant in hard coral tissue and turf algae.

Zooxanthellae cell density and mitotic index

Cell density of hard coral zooxanthellae (*Pocillopora* cf. *verrucosa*) doubled over the 8 weeks, while their mitotic index halved (Figure 9.4A and Figure 9.4B). However, zooxanthellae density and mitotic index in fertilized and control corals remained similar. After 8 weeks, cell densities ranged from 1.324 ± 0.147 Mio. cells cm^{-2} (treatment) to 1.373 ± 0.172 Mio. cells cm^{-2} (control), whereas the mitotic index ranged from 4.718 ± 0.445 % to 4.901 ± 0.244 % in organisms under control and N enrichment conditions, respectively.

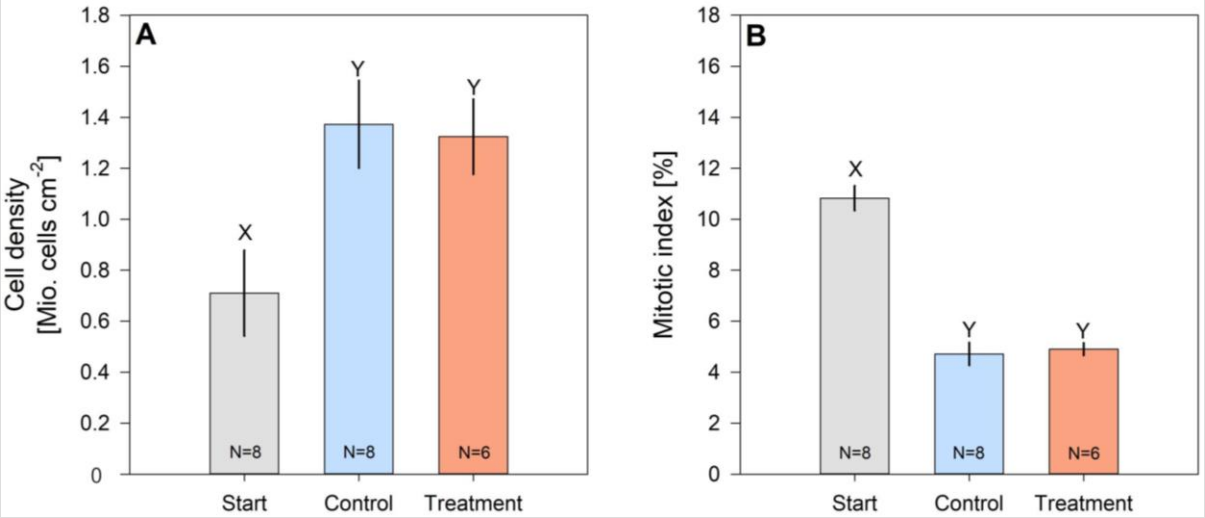

Figure 9.4 | Cell density **(A)** and mitotic index **(B)** of hard coral's (*Pocillopora* cf. *verrucosa*) zooxanthellae before (grey), without (blue) and with 8 weeks of N eutrophication (red). Error bars represent the standard error of the mean, letters X and Y indicate significant differences.

9.5 | Discussion

Studies on the effects of eutrophication in the oligotrophic central Red Sea are scarce, and have, so far, focused on eutrophication effects on single functional groups only, used natural gradients or left out impacts on the elemental stoichiometry. In a comparative in−situ approach we therefore provide an assessment of eutrophication effects on several major functional groups' ecophysiology using elemental and stable isotope analysis, drawing conclusions on N uptake and utilization.

Uptake of excess N by major benthic functional groups

In−situ N enrichment resulted in an uptake of N in the tissues of turf algae, soft corals and hard corals, but not in sediments and hard coral zooxanthellae. The fact that turf algae exhibited the lowest $\delta^{15}N$ at the

start of our experiment suggests considerable assimilation of N from N_2 fixation[77–79] (El-Khaled et al., Chapter 8 of this thesis). Biological fixation of atmospheric N_2 leads to a depletion in ^{15}N in the N compounds of the fixer[80,81]. After the experiment, turf algae showed one of the strongest uptakes of N from the fertilizer among the benthic reef functional groups, as indicated by significantly higher $\delta^{15}N$ values (Figure 9.3A), which concurs with den Haan et al.[67]. The low DIN/PO_4^{3-} ratio in ambient waters at the reef further suggests a relatively low degree of P−limitation under ambient conditions, which may help to explain the strong uptake of N when available. Concordantly, Lapointe et al.[43] showed that in the eutrophic waters of the Florida Keys, the N:P ratio of turf algae (293:1) increased to a much greater extent than that of macroalgae (71:1) as DIN concentrations increased over a 3−decade period. Also, soft corals incorporated fertilizer N and reached higher $\delta^{15}N$ values than typical for soft corals that are exposed to industrial and urban run−off in the central Red Sea[5].

In the present experiment, the uptake of excess N was not noticeable in the sediment $\delta^{15}N$, in contrast to a study by Miyajima et al.[82]. There, sediment microflora took up NO_3^- and NH_4^+ in bottle incubations[82], and assimilation as well as adsorption of N compounds on carbonate reef sands were observed[83]. This would suggest fast nutrient uptake, especially in microalgae on the sediments, and high uptakes into pore water, as reported by Rasheed, Badran & Huettel[84] and Erler, Santos & Eyre[83]. Pore water was not targeted in our study and a change of isotopic signature in the sediment could take longer than the current experimental period, as previously suggested by Cook et al.[85] who did not find clear $\delta^{15}N$ patterns in N manipulated sediments. This would account for the integration time of isotopic signatures through the food−web[86,87]. In agreement to our study, Koop et al.[48] also did not find high $\delta^{15}N$ uptake in sediments. Potentially, organisms on and in the sediments are not N−limited, given that sediments are active sites of microbial N transformations[88] and remineralization[89] allowing for N recycling.

Within the hard coral holobiont, the zooxanthellae did not incorporate excess N significantly. This generally agrees with Koop et al.[48] and Den Haan et al.[67] showing that hard corals (i.e., *Madracis mirabilis* (now *Madracis myriaster*)) take up less excess nutrients than turf or macroalgae. Most studies, however, report stronger $\delta^{15}N$ enrichment in the zooxanthellae fraction compared to host tissue[90–92]. There are several possibilities why this was not observed in our study. Firstly, NO_3^- uptake in zooxanthellae can be highest under low NH_4^+ availability[93,94], however, NH_4^+ was elevated ~5−fold compared to the environmental background in our experiment (Supplementary Table S9.1). Secondly, P can be a limiting factor to zooxanthellae's N uptake[95]. Unlike the highly increased N availability, the P provided by our fertilizer did not alter the PO_4^{3-} concentration 25 cm away from the source (Supplementary Table S9.1). The resulting increased DIN/PO_4^{3-} ratio at the communities underlines a stronger P−limitation under manipulation. Potentially, reef sediments[96] or organisms in the water column and the surrounding benthos took up PO_4^{3-} too quickly as P was stated crucial[97] and limiting[5,74,98] for primary production in coral reefs. However, we acknowledge dissimilar findings on the limiting roles of N and P in the central Red Sea[13]. The understanding of P cycling and limitation in coral reef environments is still in its infancy[99], but PO_4^{3-} could have been limiting for significant N uptake in zooxanthellae[95]. In contrast to other findings[93,100], coral tissue incorporated more available N than the symbionts. This suggests that the host tissue was less P−limited than the zooxanthellate fraction, and hence took up relatively more N. This is corroborated by the low DIN/PO_4^{3-} ratio of 3.4 in ambient waters of the studied reef that indicates N−rather than P−limitation, confirming Al−Farawati, El Sayed & Rasul[101].

Utilization of excess N by benthic functional groups

Due to eutrophication, tissue N content significantly increased in turf algae and soft corals but not in hard corals and sediments. C_{org} content remained constant in hard and soft corals but increased in sediments and turf algae. Thus, turf algae and hard coral tissue remained at constant C_{org}/N ratio, while it decreased in soft corals and showed unclear trends in hard coral zooxanthellae and sediments.

For turf algae, N and C_{org} content were significantly higher under nutrient addition compared to controls, which contrasts findings by Stuhldreier et al.[40] reporting no such eutrophication effects on turf algae dominated settlement communities. In the present study, relatively similar increases in N content (+39 %) and C_{org} content (+33 %) occurred and the C_{org}/N ratio stayed constant (between 9.1 and 9.3). Hence, we interpret N to be a limiting nutrient[102] for turf algae growth, which also corroborates other studies[56,103,104]. Turf algae are strong opponents to corals[6,105], and their competitiveness under high−nutrient availability has been documented in Hawaii[106], the Caribbean[107], Brazil[108], Australia[109] and in the Red Sea[15]. As turf algae are also rapidly taking over bare substrates[6,40] and are very resistant to disturbances[105], their monitoring should be on regional management agendas. Cover data was not documented in the present study but turf algae growth could be speculated upon based on increases in C_{org} content.

Nitrogen was taken up by soft corals while the C_{org} content did not increase, resulting in an altered elemental stoichiometry (C_{org}/N ratio). The strong decline in the soft corals' C_{org}/N ratio could be explained by an uptake of excess N as so−called "luxury consumption"[38], describing on−going uptake while a different nutrient (e.g., P) might limit growth and productivity. We interpret that in our eutrophication experiment P rather than N was the limiting nutrient for soft corals, which may limit chlorophyl *a* content and photosynthesis in Xeniidae[110]. Our data from elemental analysis and non−documented visual observations support the hypothesis of Fabricius et al.[111] that soft corals could react timelier and strongly to water quality gradients than hard corals.

Our results further correspond to other studies[84,88,112] finding low C_{org} content in carbonate dominated reef sands (0.18–0.36 %), which were even lower in our study (0.1 %). We acknowledge that the utilized acid wash−out processing may underestimate C_{org} contents due to C_{org} losses to the liquid acid of 4–52 %[113] and as such lower the C_{org}/N ratio. However, this is a commonly used method in comparative studies[84,112]. The observed 21 % increase in C_{org} could be attributed to P−supported algae growth on the sediments (Supplementary Figure S9.1) as fertilizer N was not taken up (constant $\delta^{15}N$) but gross primary production significantly increased (El-Khaled et al., Chapter 8 of this thesis). A different source for the increased C_{org} content in sediments could be the export of C_{org} from turf algae (Roth et al. in review) for example, as dissolved organic carbon (DOC)[114] and subsequent uptake by reef sediments, as suggested by Cárdenas et al.[115]. This, along with the low C_{org}/N ratio, corroborates the previous assumption that life in and on the sediments, as well as its increase in C_{org} content was not N limited.

Regarding the hard coral holobiont, our results suggest that the incorporation of excess N only to the host tissue did not result in its utilization in terms of C_{org} production. As such, C_{org}/N ratios for *Pocillopora* cf. *verrucosa* contrast a study conducted in 10 km distance to our study site further offshore at the same time of the year in 2012[116]. There, the natural host total C:N ratio was around 5, which makes our presented host material appear more N depleted in comparison. Over time, we observed an increase in symbiont cell density, which contrasts with other studies finding higher zooxanthellae cell densities in *Pocillopora* species in cooler and more nutrient rich phases[117–119]. However, particularly the similarity between treatment and non−fertilized controls should be considered where cell density and

mitotic index did not differ. Similar findings have been reported by Ferrier−Pagès et al.[120] and Rosset et al.[121] during pure N fertilization but contrast with other studies[122–124]. Increased zooxanthellae cell density in hard corals was found, for example, after only 18 days of eutrophication[125] or following a natural nutrient gradient[126]. Altogether, this suggests that N was not a limiting factor for zooxanthellae in our experiment. The production[127], health and density[94] of zooxanthellae cells was found to be P limited. Accordingly, high P availability resulted in higher increases of zooxanthellae density (*Pocillopora damicornis* and *Euphyllia paradivisa* (now *Fimbriaphyllia paradivisa*)) than availability of only N[121,122]. As a consequence, we hypothesize that hard corals also did not shift in primary productivity, even though a significant increase in $\delta^{13}C$ in hard coral zooxanthellae (Supplementary Figure S9.2) could be a sign of increased photosynthesis[128] for example, following a seasonal pattern (Roth et al., in review). However, gross primary production did not increase in our manipulated hard corals (El-Khaled et al., Chapter 8 of this thesis). The increased $\delta^{13}C$ in the zooxanthellae (Supplementary Figure S9.2) could also be an indicator for a negative effect on hard coral health which was also found in relation to bleached *Favia favus* (now *Dipsastraea favus*) corals in the Northern Red Sea[129] but not *Montastraea faveolata* (now *Orbicella faveolata*) in Florida[130]. Given our 8−week observation period and a comparatively cold-water temperature, our study did not provide a setting to trace severe bleaching effects and for the Southern Red Sea it was speculated that higher nutrient availability might even benefit *P. verrucosa* to resist higher water temperature[119]. This corroborates that effects of eutrophication on coral health are not always negative[126,131,132] and do not necessarily harm or kill individual coral colonies but get outcompeted or overgrown over time[124]. Longer (3 years)[54], and both longer and stronger (1 year, 36.2 µM NH_4^+)[48] N manipulation could, however, lead to increased coral mortality[48]. In particular, reviewed findings[133], natural long−term observations[43] and laboratory experiments[34,121] with high N (>3 µM and 38 µM N, respectively) and low P supply (<0.07 µM and 0.18 µM P, respectively) increased susceptibility of corals to bleaching, which suggests negative effects. In agreement with Ezzat et al.[127] and Ferrier−Pagès et al.[99], we suggest increasing efforts investigating P cycling and limitation in current and future reef ecosystems. Besides this key role of nutrient ratios, Burkepile et al.[32] highlight the importance to also account for varying effects of different forms of N. As N sources and pathways in corals and their reef environments are of major importance to better understand ecosystem functioning[134], the uptake and utilization of N (this study) should be compared to eutrophication effects on the N cycle.

Conclusions

Anthropogenic pressures on the Red Sea are constantly increasing[135] and 60 % of Red Sea coral reefs are at stake[136]. We were able to show cascaded, group−specific responses to N availability and link elemental and isotopic composition to group−specific nutrient limitations, N uptake and utilization, and highlight the importance of P limitations in hard and soft corals. Even over an 8−week N eutrophication and under high abundance of herbivores, significant uptake and utilization of fertilizer N was shown particularly for turf algae as strong competitors for space in struggling reef ecosystems. As such, our study corroborates that turf algae can be early indicators for changes and anthropogenic influence[137,138], reacting faster to eutrophication than hard coral zooxanthellae. As turf algae play a key role in phase shifts, are strong competitors to corals, rapidly take over bare substrates and are highly persistent, their substantial biochemical benefits from N supply should push coastal management to not only consider limiting future discharges but try to reduce both point−sources and non−point sources of nutrients already in place. Given

the increasing coastal development in the central Red Sea, water quality management is challenged to improve future reef states[139,140] and should be on regional agendas for coastal urban development and aquaculture. The context in which eutrophication effects should be seen comprises further local (e.g., fishing pressure and habitat destruction) and global (e.g., warming and ocean acidification) factors to which coastal development adds high nutrient loads on top. Low N concentrations were shown to be a crucial precondition for coral recovery[141] and particularly in the Red Sea the maintaining of oligotrophic conditions could be the key factor and challenge for coral health and resilience to climate change[22].

9.6 | Acknowledgments

We are thankful to Marianne Falk for helping in elemental−and isotopic analysis and Nils Rädecker for coral sample processing advice. We were happy to get further support by Pedro Ruiz−Compean (sediment samples), Aislinn Dunne (sediment samples), Sophia Tobler (image editing), Rodrigo Villalobos and João Cúrdia (both field work). This research was supported by DFG grant Wi 2677/9−1 to Christian Wild, KAUST baseline funding to Burton H. Jones and the KAUST VSRP program to Denis B. Karcher. Susana Carvalho is funded by the Saudi Aramco−KAUST Center for Marine Environmental Observations. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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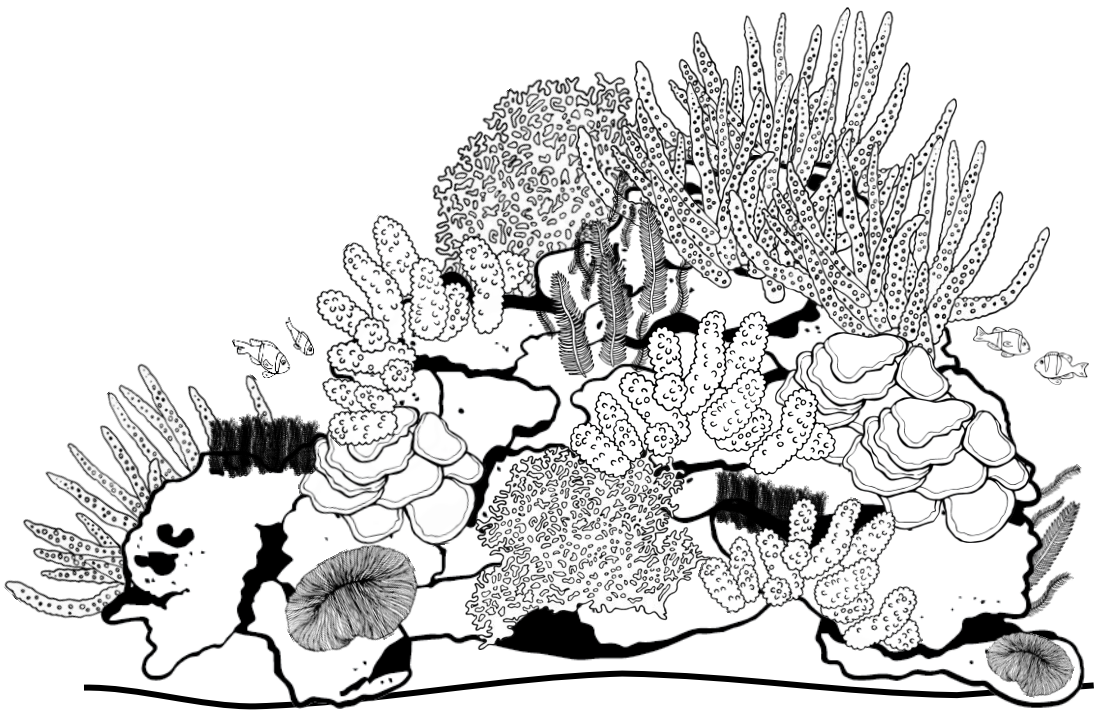
Chapter 10



Chapter 10 | **Prospective phase shifts from corals to algae domination promote nitrogen accumulation in reefs**

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10.1 | Abstract

Coral reefs worldwide have experienced phase shifts from coral– to algae–dominated benthic reef communities. The pace and degree of such shifts is affected by nitrogen (N) availability, which in turn is partly a consequence of biological dinitrogen (N_2) fixation and the antagonistic process denitrification. There are pronounced gaps of knowledge related to N cycling in coral reefs in general, and in particular how phase shifts affect this key biogeochemical cycle. Therefore, we assessed N_2 fixation and denitrification in parallel for six key benthic functional groups (hard corals, soft corals, turf algae, coral rubble, biogenic rock, and reef sands with their microbial communities) that, together, accounted for > 98 % of the benthic cover in the investigated central Red Sea coral reef. Measured rates using a combined acetylene assay were extrapolated to the relative benthic cover of the studied organisms in coral– and algae–dominated reef areas. Findings revealed that functional groups associated with high N_2 fixation (turf algae and coral rubble) exhibited low associated denitrification rates. Soft corals displayed up to six–fold higher denitrification rates compared to all other groups. Extrapolated to both reef areas, turf algae and biogenic rock accounted for 75 % of overall N_2 fixation, whereas (hard and soft) corals with associated microbes contributed to 50–75 % of denitrification. Total fixed N input was two–fold higher in algae– compared to coral–dominated reefs, whereas N removal via denitrification was similar. We conclude that algae–dominated reefs likely promote accumulation of N. This could support higher net productivity, potentially resulting in a functional advantage compared to coral dominated reefs.

Keywords: Dinitrogen fixation | denitrification | diazotrophy | nutrient cycling | biogeochemical cycling | Red Sea | Nutrient Budget | Metabolism

A modified version of this chapter will be submitted to the journal *Ecosystems*

10.2 | Introduction

Nitrogen (N) is vital for all living organisms and is required for primary production and building biomass. Among the key elements (i.e., N, carbon, phosphorus, oxygen and sulphur)[1], N has the greatest total abundance in the environment[2]. Ironically, however, it is the least accessible for flora and fauna[1]. Particularly in oligotrophic marine ecosystems such as coral reefs, primary production is limited by low amounts of bioavailable N[3,4]. Although flourishing in oligotrophic environments and being N–limited[5–7], coral reefs belong to the most productive ecosystems on earth and are regarded as oases in an oceanic desert[8–10]. Here, microbial N–cycling is thought to play an important role. Especially biological dinitrogen (N_2) fixation, i.e., the conversion of atmospheric N_2 into bioavailable ammonium (NH_4) by prokaryotic microbes (diazotrophs), potentially alleviates N limitation for marine primary producers[11]. Contrary to this, denitrification (i.e., the conversion of nitrate to atmospheric N_2 by microbes) can serve as a counteracting pathway within the N–cycle, potentially relieving N in times of high environmentally offered N[12–14]. Whereas N influxes to coral reefs via N_2 fixation are comparably well–studied over decades[4,15–18], knowledge about N efflux via denitrification is limited for coral reef substrates[12,13] and just starting to be generated for coral reef organisms[19–22]. Hypothetically, the interplay of both pathways is key to sustain the productivity of coral reefs and, hence, ecosystem functioning[23].

Coral reefs not only belong to the most productive, but also to the most threatened ecosystems on the planet. Global and local change associated stressors such as ocean warming and acidification, eutrophication, and overfishing[24,25] undermine the health of coral reefs and can eventually lead to coral die–off[26]. Once dead, whole reef areas offer substrates[27–29] for fast–growing, highly competitive algae assemblages. This process has been described as a regime– or phase–shift from coral–dominated to algae–dominated reef states[30–32]. The likelihood of such shifts is even enhanced through elevated nutrient concentrations and the absence of herbivorous fishes caused by e.g., overfishing[31,33,34]. With predictions that mass coral bleaching events will occur more frequently[35,36], coral reef recovery times reduce significantly[37] as well as the return to coral–dominated states[38]. Although the effects of these regime–shifts on ecosystem services[39–43] and functioning[44,45] have received some attention, Williams & Graham[46] emphasise the dimensions of our yet rudimentary understanding of alterations in coral reef functional ecology. This is critical, as we yet face coral reefs in different states (i.e., coral–dominated or alternative states), with knowledge about the functioning of these states still being in its infancy. Even beyond, functioning is likely to differ between coral– and algae–dominated communities[47], and this information is crucial to understand the stability and trajectory of these novel communities.

N cycling is of key importance for the stability of coral reef ecosystems; however, it has not been investigated yet, how coral– and algae–dominated reefs differ quantitatively regarding their N–cycling processes. From variable N–flux capacities of different functional groups[11,17,48] and an altered reef community structure associated with regime–shifts, we hypothesise changes in reef–wide N dynamics and the overall budget. Potentially, N–cycling processes have the potential to even amplify and catalyse regime–shifts through the dominating role of turf algae in regime–shifts[49], their strong inherent N_2 fixation capacities in reefs[11] and the foundation of new communities in particular. The role of denitrification in reef communities is largely unknown, as well as the interplay of both N_2 fixation and denitrification in coral– and algae–dominated reefs. Understanding N–cycling patterns in baseline scenarios (i.e., coral–dominated) and alternative reef states (i.e., algae–dominated) is, hence, of paramount interest to gain a

holistic understanding of these dynamic systems, which then can be used as a basis to address, elaborate and expand future management strategies.

Thus, in the present study, we carried out acetylene–based short–term incubations (*i*) to identify the key players that import and/or relieve nitrogenous compounds into or from the reef system; (*ii*) to provide a relative budget for two antagonistic N–cycling processes (i.e., N_2 fixation and denitrification) in a comparative framework that covers > 98 % of the benthic functional group cover on a central Red Sea coral reef; (*iii*) to relate N–cycling rates of all dominant functional groups with their relative cover in a coral–dominated and an algae–dominated reef area.

10.3 | Materials and Methods

Study site and benthic community composition

The Abu Shosha reef in the Jeddah Region (N22°18`15", E39°02`56") on the west coast of Saudi Arabia in the central Red Sea was chosen due to the presence of both coral– (i.e., > 40 % hard coral cover) and algae (< 15 % hard coral cover, > 40 % turf algae cover) dominated reef areas[50]. Both areas were located at the same water depth (~5 m). Currently, the reef experiences a phase–shift transition resulting in a small–scale heterogeneity of communities in varying degrees of community transitions. For this reason, this particular reef was selected with both target reef areas being less than 30 m apart from each other.

Benthic community composition of the Abu Shosha reef was determined for an earlier study[50] by photo quadrats[51], providing a two–dimensional (2D) planar reef coverage of each functional group. Briefly, a PVC quadrat (50 x 50 cm², 0.25 m²) was randomly placed on the reef surface (12 x in coral–dominated area, 10 x in algae–dominated area), and a photograph was taken from approx. 1 m distance to the substrate. Photographs were then analysed with the software Coral Point Count with Excel extension (CPCe) 4.1[52]. With the help of the software, 48 randomly located points were overlaid on the photographs, resulting in 480 data points per study area. The underlying benthos under each data point was determined to the lowest possible taxon. The major functional groups were: hard corals, soft corals, macroalgae, filamentous turf algal assemblages (hereafter termed turf algae), biogenic coral rock, coral rubble, carbonate reef sands, and the giant clams (*Tridacna* sp.). Hard and soft corals were identified to the genus level. Turf algal communities consisted of a heterogeneous assemblage of filamentous algae including cyanobacteria. Areas of bare coral rock, that were not covered with any of the other functional groups but associated with endolithic algae and crustose coralline algae were defined as biogenic coral rock (hereafter biogenic rock)[53]. Coral rubble was defined as dislodged parts of framework builders with its associated microbial community according to Rasser & Riegl[54].

Sample collection and maintenance

All benthic functional groups, i.e. hard corals of the genus *Pocillopora verrucosa* (n = 5), *Acropora hemprichii* (n = 4), *Stylophora pistillata* (n = 4), soft corals of the *Xenia* genus (n = 5), biogenic rock (n = 5), coral rubble (n = 4), reef sands (n = 5) and turf algae (n = 5), were collected randomly from the overall reef area (i.e., regardless whether from coral– or algae–dominated areas). The above functional groups were selected as they comprised more than 98 % of the benthic cover in both coral– and algae–dominated parts of the reef[50]. Where necessary, fragments were collected with hammer and chisel. *P. verrucosa*, *A. hemprichii*, *S. pistillata* and turf algae fragments (limestone covered with turf–algae) were approx. 10 cm long. Hard coral fragments were sampled from different coral colonies (> 10 m distance between each

other) to account for genetic diversity. Individual coral colonies of *Xenia* sp. were collected with a small piece of anchoring rock (< 0.5 cm diameter) to prevent tissue damage. Reef sands were sampled using a Petri dish (polystyrene, 5.5 cm diameter, 1.4 cm depth) which was pushed carefully into the sand. Reef sands were then fixed to the dish from underneath so that upper sand "cores" with a max. sediment depth of 14 mm were sampled. Surface sediment layers were chosen as these exhibit a relatively high bacterial activity (and, thus, likely higher biogeochemically active bacterial communities) compared to deeper layers[55]. All fragments and Petri dishes containing reef sand samples were immediately transferred to recirculation aquaria on the boat after sampling, each filled with ambient seawater. Fragments of *P. verrucosa*, *Xenia* sp., turf algae, coral rubble, biogenic rock as well as sediment samples were kept at ambient water temperature and light conditions until the experimental incubations started within 3 h after sampling. Fragments of *A. hemprichii* and *S. pistillata* were transferred to the wet lab facility of the Coastal and Marine Resources (CMOR) Core Lab at KAUST and were distributed into four independent replicate 150 L flow–through tanks (flow–through rate 300 L h^{-1}, with ambient reef water and ambient light conditions) for two weeks to allow for acclimation and healing of tissue damage.

Nitrogen fluxes

Incubations were performed using a COmbined Blockage/Reduction Acetylene assay (hereafter COBRA) modified after El–Khaled et al.[56]. Briefly, COBRA incubations were performed in gastight 1 L glass chambers (800 mL seawater + 200 mL headspace). As acetylene inhibits the production of nitrate via nitrification[57,58], seawater was supplemented with nitrate to a final concentration of 5 µM as a substrate for the denitrification pathway to counteract substrate limitation[59–61]. Freshly generated acetylene was added to both incubation water and headspace at a concentration of 10 %. Providing acetylene in the gastight incubation chambers leads to the preferential reduction of acetylene to ethylene (C_2H_4) instead of N_2 to NH_4 by the key enzyme nitrogenase[62,63]. Moreover, acetylene blocks nitrous oxide (N_2O) reductase activity in the denitrification pathway leading to an accumulation of N_2O[64,65]. Replicate samples were incubated and two additional chambers without specimens served as controls to correct for planktonic background metabolism. All incubations lasted for 24 h with a 12:12 h dark/light cycle and a photon flux of ~200 µmol quanta m^{-2} s^{-1}. Incubation chambers were submersed in a temperature–controlled water bath (similar to ambient seawater temperature: 27 °C) and constantly stirred (500 rpm) to ensure sufficient exchange between water body and headspace. Gas samples were taken at the start (t_0) and the end (t_{24}) of each incubation, and analysed targeting C_2H_4 (as a proxy for N_2 fixation) and N_2O (as a proxy for denitrification) by gas chromatography and helium pulsed discharge detector (Agilent 7890B GC system with HP–Plot/Q column, lower detection limits for both target gases were 0.3 ppm). Results were then normalised to incubation time, corrected for the seawater control signal, related to incubation volume and normalised to surface area of the organisms/substrates. Surface areas of incubated organisms/substrates were determined photometrically using cloud–based 3D models (Autodesk Remake v19.1.1.2)[66,67] of *P. verrucosa*, *A. hemprichii*, *S. pistillata*, *Xenia* sp., biogenic rock, coral rubble and turf algae fragments. Reef sand surface areas were calculated using dimensions of Petri dishes that were utilised for sand core sampling (surface area = π * $radius^2$).

Data treatment of N fluxes

Production rates of C_2H_4 and N_2O were converted into N production using molar ratios of $N_2O:N_2 = 1$ and $C_2H_4:N_2 = 4$[56]. At this stage, individual rates are presented for each respective functional group that was collected randomly from the overall reef area. Detected rates (expressed h^{-1}) were then transformed into daily N fluxes for each functional group. Subsequently, daily rates were extrapolated according to reef benthos 3D area considering the respective 2D:3D conversion factor (Supplementary Table S10.1)[11]. This represents daily N_2–fix and DENI fluxes, extrapolated to a theoretical square meter of reef benthos entirely covered by the respective functional group. Finally, these functional group–specific rates were used to account for the relative cover (i.e., 2D planar coverage obtained from cover assessments described previously) of each functional group in a respective reef area (i.e., coral– and algae–dominated, resp.) which provides N fluxes related to 3D reef area (expressed as fixed or denitrified mg N m^{-2} benthos 3D area d^{-1}, resp.).

Statistical analysis

Statistical analyses were performed using Primer–E v6[68] with the PERMANOVA extension[69]. Differences in the N–cycling processes among groups and related to both reef areas were tested for significance using permutational analysis of variance (PERMANOVA) on a Bray–Curtis similarity matrix of square–root transformed data. Type I (sequential) sum of squares was used with a permutation of residuals under a reduced model (999 permutations). Unless mentioned otherwise, hard coral data consists of pooled replicates of the three investigated hard coral species (i.e., *P. verrucosa*, *A. hemprichii*, *S. pistillata*) of which mean rates and standard error of means were calculated. Normality (Shapiro–Wilk test) and differences between functional group coverage in both reef areas (e.g., hard coral cover in coral–dominated versus hard coral cover in algae–dominated areas) were tested using SigmaPlot (Version 12.0). Two–tailed t–tests were used if data were distributed normally whereas Mann–Whitney–Rank–sum tests were used if data were not normally distributed. Differences among reef areas (i.e., coral vs. algae–dominated) were visualised using a principal coordinate analysis (PCO). A one–way analysis of similarities (ANOSIM; 999 permutations) was used to describe the dissimilarities between both reef areas. Furthermore, a distance–based linear model (DistLM; 999 permutations) using a step–wise selection procedure with AICc as a selection criterion was used to calculate which functional group(s) coverage explained visualised dissimilarities best[68,69].

10.4 | Results

Benthic community composition

Two distinct reef community states characterised by contrasting relative cover of functional groups were identified (Figure 10.1A). A detailed overview of the benthic communities is attached as Supplementary Table S10.2. Briefly, algae–dominated areas displayed twice as much algae–cover (combined turf and macroalgae cover) compared to coral–dominated areas ($p = 0.007$), whereas hard coral cover was 3–fold lower ($p < 0.001$). Separations were confirmed visually by Principle Coordinate Analysis (PCO, Figure 10.1B), identifying a clear distinction between both reef areas (ANOSIM, $R = 0.605$, $p = 0.001$). Indeed, hard corals and turf algae were the major drivers of separation and explained 64.2 % of dissimilarities between both reef areas (DistLM).

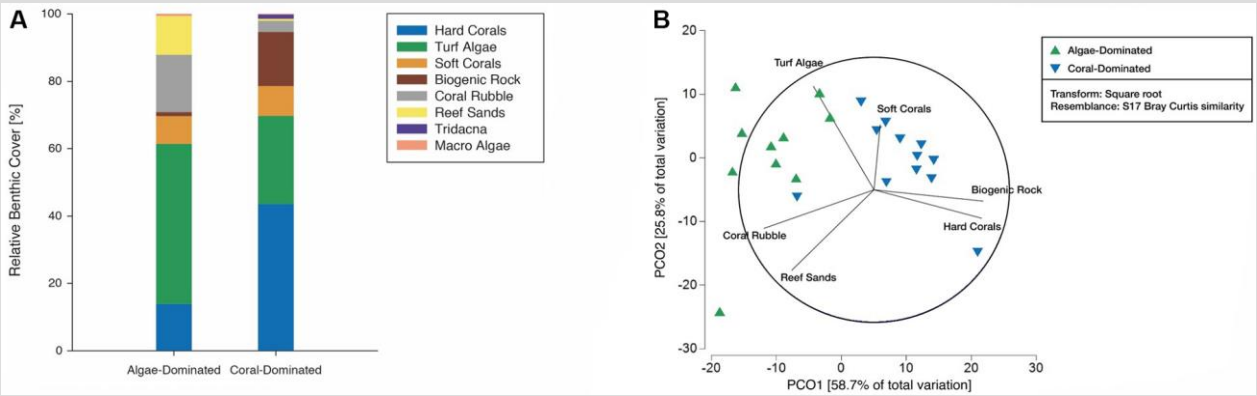

Figure 10.1 | **(A)** Relative benthic cover in algae– and coral–dominated areas. Benthic cover data is presented as mean proportional cover of major benthic functional groups assessed by photo quadrats (n = 10 in algae–dominated areas, n = 12 in coral–dominated areas). **(B)** Principle Coordinate Analysis (PCO) of benthic community cover at the algae–dominated (green) and coral–dominated (blue) areas. Vector overlay shows correlations > 0.4 based on Pearson ranking.

N fluxes of individual functional groups

Turf algae and coral rubble showed highest N_2 fixation rates per 2D surface area, being 5–fold higher than those of biogenic rock, 10–fold higher than reef sands, ~35–fold higher than for soft corals and approx. two orders of magnitude higher than for hard corals (Figure 10.2A, Table 10.1, Supplementary Table S10.3). Soft corals displayed highest denitrification rates that were 3– to 4–fold higher than those of reef sands, biogenic rock and coral rubble, and 6–fold higher than denitrification rates of hard corals and turf algae (Figure 10.2B, Table 10.1, Supplementary Table S10.3). Among hard corals (i.e., data above comprised the rates for *P. verrucosa*, *A. hemprichii*, and *S. pistillata*), *A. hemprichii* showed highest denitrification rates that were 5–fold higher than those of *P. verrucosa* (pair–wise PERMANOVA t = 3.407, $p = 0.01$) and 26–fold higher than *S. pistillata* (pair–wise PERMANOVA t = 5.696, $p < 0.001$).

Table 10.1 | Results (top: t–values; bottom: *p*–values) of permutational analysis of variance (PERMANOVA) pair–wise tests for N_2 fixation (highlighted in salmon) and denitrification (highlighted in blue) in all functional groups.

	Turf Algae	Coral Rubble	Soft Coral	Biogenic Rock	Reef Sands	Hard Corals	
Turf Algae		0.882 0.416	8.793 **0.001**	6.774 **0.001**	10.773 **0.001**	7.439 **0.001**	DINITROGEN FIXATION
Coral Rubble	1.814 0.13		6.913 **0.001**	4.690 **0.004**	7.463 **0.001**	6.362 **0.001**	
Soft Coral	4.400 **0.003**	2.825 **0.020**		4.637 **0.001**	3.299 **0.003**	2.374 **0.030**	
Biogenic Rock	1.473 0.154	0.186 0.893	2.869 **0.018**		2.550 **0.031**	5.501 0.001	
Reef Sands	1.344 0.177	0.218 0.867	2.643 **0.025**	0.080 0.970		4.651 **0.001**	
Hard Corals	0.944 0.350	1.669 0.112	3.585 **0.001**	1.690 0.100	1.643 0.104		
	DENITRIFICATION						

Significant *p*–values in bold.

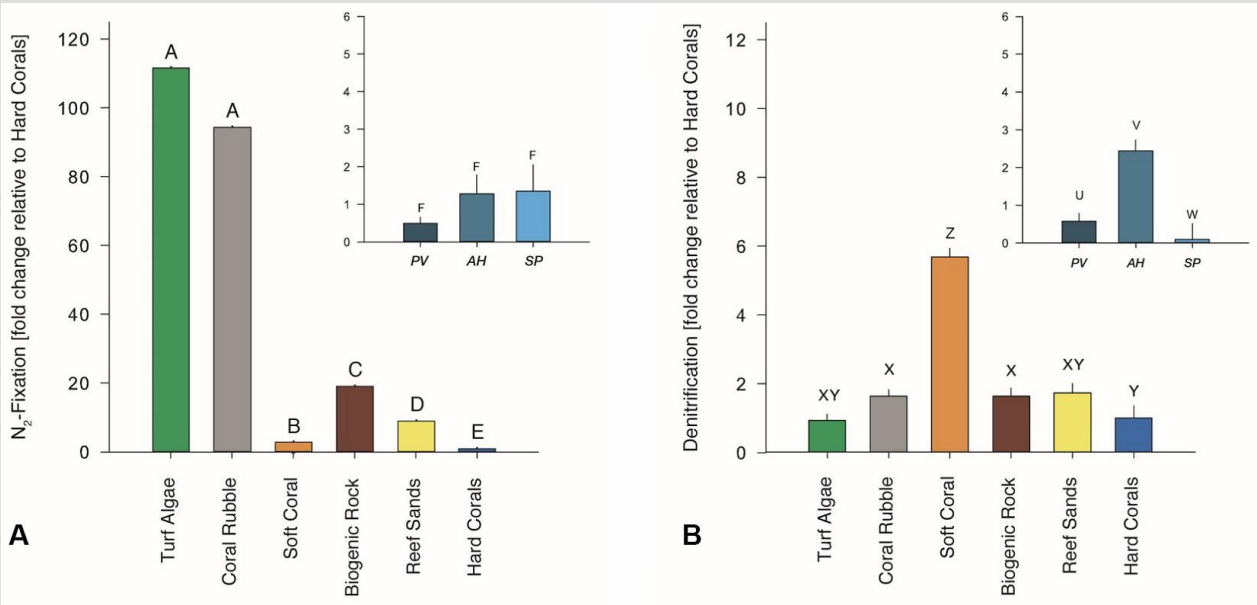

Figure 10.2 | Dinitrogen (N_2) fixation **(A)** and denitrification **(B)** rates of investigated functional groups related to mean N_2 fixation/denitrification rates of hard corals (5.11 ± 1.89 and 6.47 ± 2.27 pmol N_2 cm^{-2} h^{-1}, respectively) ± relative standard errors. Organisms and substrates (turf algae n = 5, coral rubble n = 4, soft coral n = 5, biogenic rock n = 5, reef sands n = 5, hard corals n = 13) were sampled randomly from both reef areas. Rates for hard corals consist of mean values of *P. verrucosa* (PV, n = 5), *A. hemprichii* (AH, n = 4), and *S. pistillata* (SP, n = 4), which are shown in individually implemented graphs. Letters above bars indicate significant differences if different, or non–significance if shared.

N fluxes in different functional groups referred to reef areas

We extrapolated individual rates of N_2 fixation and denitrification from all measured organisms and substrates (see Figure 10.2) to produce budgets for communities of the assessed reef areas dominated either by corals or benthic turf algae. Total fixed N was 2–fold higher in algae–dominated compared to the coral–dominated area, whereas denitrification remained similar (Figure 10.3A). Turf algae assemblages contributed most to N_2 fixation in both algae–dominated (70.2 %) and coral–dominated reef areas (79.8 %), followed by coral rubble, which account for 28.4 % in algae–dominated and 11.3 % in coral–dominated reef areas (Figure 10.3B). In contrast, corals (hard and soft corals combined) accounted for 77.6 % and 54.3 % of denitrification activity in coral– and algae–dominated areas, respectively.

10.5 | Discussion

The presence or absence of N is of central importance to the health and resilience of coral holobionts[14,70], and, consequently, of coral reef ecosystems in general[23]. Thus, processes that introduce or relieve N, such as N_2 fixation and denitrification, may play a key role for the functioning of coral reefs[23]. Hence, a detailed overview of both these N–cycling processes for key coral reef functional groups is required. The present study extends the previous work of Cardini et al.[11], who showed a link between reef–wide primary production and N_2 fixation, by adding a further N–cycling process (i.e., denitrification). We were able to relate substrate/organism–specific N_2 fixation and denitrification rates (per 2D substrate surface area) to their extrapolated relative contribution on a 3D level, and likewise extrapolate both metabolic processes

to both coral– and algae dominated reef communities (per 3D benthos surface area). The latter are expected to increase in number as a consequence of frequently occurring mass coral bleaching events[35,36] and reduced recovery times[37] that diminish the return to coral–dominated reef states[38].

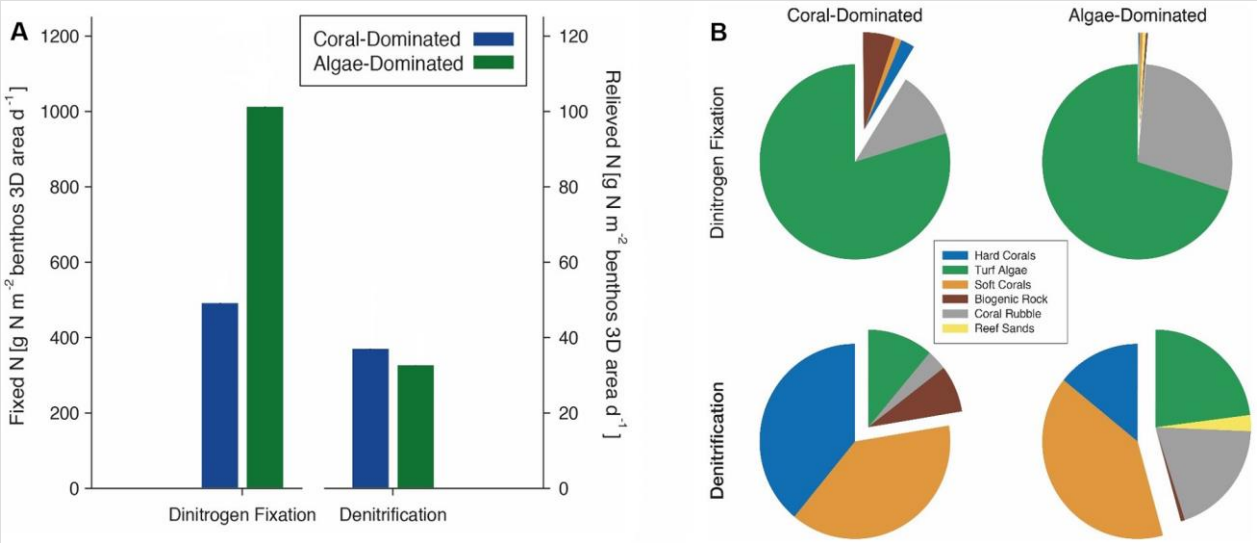

Figure 10.3 | **(A)** Total fixed (via dinitrogen fixation) and relieved N (via denitrification) in both reef areas (calculated as sum of means of extrapolated rates of individual functional groups). **(B)** Relative contribution of studied functional groups to dinitrogen fixation (top pie charts) and denitrification (bottom pie charts) budget in coral– (left pie charts) and algae–dominated (right pie charts) reef areas.

Facilitation of N–cycling processes by key functional groups

Individual N_2–fix rates of respective functional groups are in line[11,17,22,48,71] or marginally lower[11,72] than rates reported in previous studies from the Red Sea and that were obtained with similar measurement techniques. Slightly lower N_2 fixation rates in the present study may be due to an underestimation caused by an initial lag phase associated with acetylene–based assays[73,74], which was omitted from rate calculations in mentioned studies. However, underestimations in the present study appear less significant for N_2 fixation rates than for denitrification, for which a relative comparison is still possible[56,75].

The results suggest a clear distinction between key functional groups, in which the most active N_2–fixers showed lowest denitrification rates and *vice versa*. Turf algae and coral rubble and their associated microbial communities were identified as the strongest N_2–fixers confirming previous findings by Cardini et al.[11]; however, they play a minor role in denitrification activities. Reef sands as active sites of microbial N transformations in coral reefs[12], play only a marginal role in both N_2 fixation and denitrification in the present study, compared to the other substrates. Potentially, comparatively low N_2 fixation and denitrification activity is due to well oxygenated sediment layers[55] thwarting these rather anaerobic processes[76,77]. Soft corals showed comparatively low N_2 fixation activity but were the most preeminent denitrifiers among all investigated functional groups.

From an ecological perspective, turf algae are pioneers and opportunists[78,79], which can form extensive mats even under reduced nutrient–loading rates[80]. Furthermore, they can rapidly take–over bare substrate due to fast regeneration abilities[79,81,82]. The high N demand to fuel metabolism and biomass

production can, hypothetically, be satisfied by high N_2–fixing activities[17,83,84]. High N_2 fixation activity in coral rubble in the present study may be driven by microbial communities inhabiting the surfaces of coral rubble, as suggested by Davey et al.[85], who observed significantly higher N_2 fixation activity in coral rubble compared to living hard corals.

Among all functional groups, corals (both hard and soft corals) showed lowest N_2 fixation activities. However, soft corals displayed highest denitrification activity. The interplay of N_2 fixation and denitrification, favouring low internal N availability, may be vital for the stability between the coral host and the associated photosynthetic algal endosymbiont of the family Symbiodiniaceae[14]. Low N_2 fixation and comparatively high denitrification activity may promote host control of symbiotic algae[86] by keeping N availability desirably limited[6,7]. As such, environmental stressors like eutrophication or ocean warming may influence both processes[14], leading to shifts from N to phosphorus or other micronutrient limitation[87–89], which can result in a breakdown of the symbiotic association and a loss of the symbiont[87]. Furthermore, Tilstra et al.[22] hypothesised a negative link between heterotrophic capacity and denitrification activity in coral holobionts. This link may extend to soft corals as the investigated soft coral of the family Xeniidae is a functional autotroph[90] with a low heterotrophic capacity. This could explain the observation of comparatively high denitrification activity.

Implications for coral– and algae–dominated reefs

In the present study, we quantified N cycling rates of individual functional groups (see Supplementary Table S10.3), which were then used for community–wide extrapolated areas by relating the respective rate with the relative cover in both distinct reef areas (Figure 10.1) and the respective functional groups' 2D:3D conversion factor (Supplementary Table S10.2). At the community level, the relative contribution of key functional groups varies when compared to N fluxes related to substrate surface area. For N_2 fixation, the relative contribution of turf algae surprisingly reduces by 10 % in algae–dominated areas compared to coral–dominated, whereas the relative contribution of coral rubble to total N_2 fixation doubles. This is due to the increase in benthic coverage of both functional groups. Both turf algae and coral rubble account for 91.10 % of total fixed N in coral–dominated and for 98.58 % in algae–dominated reef areas.

Conversely, both hard and soft corals are the key players in coral–dominated reef areas, in which both functional groups together account for 52.40 % of benthic cover (in the investigated Abo Shosha reef area) and contributed equally to the 77.60 % of overall denitrification. This is especially surprising, as both functional groups showed lowest (hard corals) or highest (soft corals) denitrification activity. Besides being most abundant in coral–dominated areas, hard corals (especially branching hard corals, represented by the species selected in the present study) contribute most to the three–dimensional structure (i.e., spatial complexity or rugosity) of coral reef ecosystems[91–93], which increases the relative importance of hard corals on the community and ecosystem level in the present study. A reduction of hard coral cover and, thus, spatial complexity also leads to a reduced contribution to overall denitrification (Figure 10.3B). Even in the algae–dominated reef area, both hard and soft corals still contributed to more than 50 % of overall denitrification (Figure 10.3B). The mentioned reduction or loss of spatial complexity is commonly associated with coral–phase shifts[94]. Whereas the importance of structural complexity and consequences of its loss in coral reefs has been recognised on multi–fold levels before, e.g., on fish communities[94–97], invertebrate diversity[98,99] or ecosystem services[40,93,100,101], our data further suggests profound

consequences on N–cycling dynamics. The most striking result, besides changes in the relative contribution of denitrifying corals in the respective reef areas, is the difference in total N import via N_2 fixation in both reef states. Here, the interplay of turf algae that were identified as key N_2–fixers and their higher abundance in benthic coverage leads to a 100 % increase of total fixed N in algae–dominated compared to coral–dominated areas. At the same time, denitrification as a mechanism that potentially alleviates coral reef environments from excess N[12–14,19,22] remains stable in algae–dominated reef areas. This interplay (i.e., higher fixed N inputs with stable denitrification) in algae–dominated reefs results in accumulation of N that in return can lead to a relieve of N limitation, ultimately facilitating higher net productivity and growth[102,103]. This is of special importance, as a faster accumulation of biomass compared to other reef areas (i.e., coral–dominated communities) is a functional advantage under N limited (environmental) conditions. This underlines that algae–dominated areas might be highly competitive, with a fast procession of nitrogenous compounds that can result in rapid takeover of bare substrates[50,104].

Limitations and implications for further alternative states of reef ecosystems

It is in the nature of any budget that considerable uncertainties occur, such as i) deviations from theoretical molar ratios (i.e., N_2O:N_2 and C_2H_4:N_2) in different benthic functional groups[105]; ii) the mentioned methodological underestimation of N–cycle processes[56,75,106]; iii) environmental alterations by functional groups as an effect of benthic primary productivity[107] and that can potentially be evoked by varying N–cycling activities too; and iv) potential reef–wide underestimations/omission of metabolic processes in cryptic habitats, such as cracks and crevices within the natural reef matrix, that harbour specific organisms (e.g., sponges, bryozoan, and tunicates). These organisms are generally not included in *ex situ* budget derivations. However, they can remarkably contribute to metabolic processes in coral reefs[108,109]. Nevertheless, the major results of the present study are regarded as solid and reliable, especially as the investigated functional groups cover > 98 % in both reef areas so that we consider discussed and presented data as conservative estimates.

Nevertheless, we here solely evaluate two distinct reef communities, but discontinuous shifts[110], shifts of varying intensity[111] or community shifts from reefs dominated by corals to assemblages other than benthic (turf) algae have been reported[112]. These alternative reef states, besides the shifts to algae–dominated reefs, can be of corallimorpharia, sponge, ascidian or soft coral dominance. Particularly soft coral dominance is common in the Red Sea[113,114] and elsewhere[115,116], with a prevalence of *Xenia* sp. of as much as 80 % after disturbance in some locations[112,117]. Moreover, climate change associated stressors such as ocean acidification might induce community shifts from hard to so soft coral dominance[118]. Bednarz et al.[48] suppose that soft corals may become an important player in N cycling due to their increasing dominance in benthic cover in the northern Red Sea. Based on the results of the present study, we carefully speculate that a high soft coral cover may relieve degraded reefs, or reefs in a transitional state, from excessive N. To which extent the large relative importance of *Xenia* sp. as the main denitrifying functional group, can contribute to decelerate or arrest coral reef degradation, remains, however, uncertain. Hypothetically, N limitation can be exacerbated in *Xenia* sp. dominated reef areas, as more N is denitrified than in reef dominated areas of another dominance. Essential N, which is required for building biomass and/or metabolism[1], in return has to be acquired differently in this particular case.

Coral rubble–dominated areas, particularly reef flats, are the result of fragmentation and erosion processes of nearby reef areas such as leeward slopes, resulting in mobile fragments that are unsuitable

for coral colonisation[54]. Findings of the present study confirm previous hypotheses, in which the potential of coral rubble as highly active N_2–fixers can aggravate N influxes to a destroyed or delicate reef[70,85]. At the same time, our findings indicate that the denitrifying capacity of rubble–dominated reefs is presumably lower than in healthy, coral–dominated areas, which leads us to the conclusion, that this reef state will rather compound N influxes on an ecosystem level, so that N likely accumulates.

We demonstrated that N influxes via N_2 fixation lead to an accumulation of N in algae–dominated communities, especially as denitrification, serving as a relieving mechanism, remains stable. However, further processes, including anaerobic ammonium oxidation (ANAMMOX, transformation of fixed NH_4 to elemental N_2) that potentially alleviate systems from N have been identified in many marine environments[119,120], as well as in coral microbiomes[121] and coral–reef associated sponges[19]. Cryptic habitats in coral reefs harbouring high abundances of sponges[122] may be of particular importance for the export of bioavailable N via ANAMMOX. In the light of coral–reef phase shifts and an associated loss of structural complexity, and thus, cryptic habitats, the estimated loss of these potentially important N–cycling players has to be targeted in future studies.

10.6 | Acknowledgments

We are grateful to Rodrigo Villalobos and João Cúrdia for their support during fieldwork. Many thanks also to Söphiä Tobler and Jän Krause for their support in developing the figures, as well as to Najeh Kharbatia for technical support with GC. This work was funded by the German Research Association (DFG Project: Wi 2677/9–1) to CW and King Abdullah University of Science and Technology (KAUST) baseline funding to BHJ and CRV.

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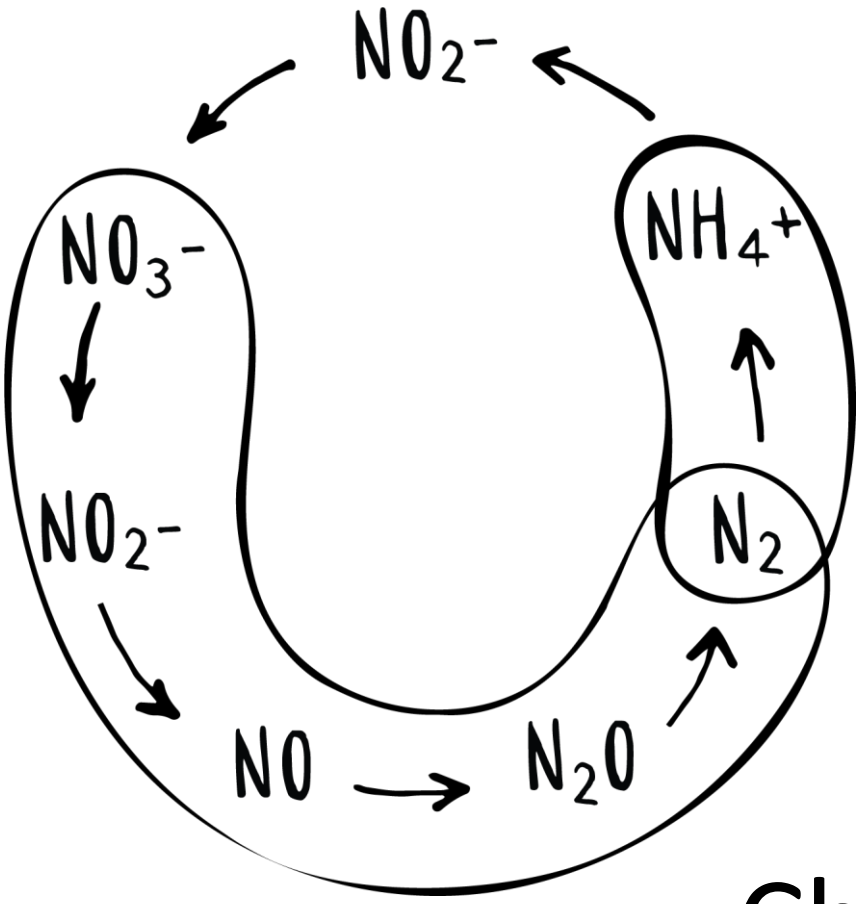

Chapter 11

Chapter 11 | **General discussion**

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Keywords: Coral reefs | nitrogen cycling | functional groups | dinitrogen fixation | denitrification | holobiont

11.1 | Thesis highlights

- Dinitrogen (N_2) fixation associated with phytoplankton directly overlaying a coral reef remains stable between two seasons (Chapter 2)[1]
- Macro- and turf algae may have an ecological advantage over corals due to higher N_2 fixation rates (Chapter 3)[2]
- Diazotrophy associated with hard corals along a depth gradient may be facilitated by the corals' heterotrophic capacity (Chapter 4)[3]
- Denitrification is an active nitrogen (N) cycling pathway in coral holobionts and aligns with N_2 fixation (Chapter 5)[4]
- The ratio of hard coral associated denitrification and N_2 fixation may dynamically shift in response to environmental dissolved inorganic N (DIN) (Chapter 7)
- Eutrophication increased denitrification, but also N_2 fixation rates associated with a hard coral, turf algae and sediments (Chapter 8)
- A eutrophication event increased $\delta^{15}N$ in hard coral tissues and soft corals but not in Symbiodiniaceae cells (Chapter 9)[5]
- Coral holobionts contributed most to denitrification activity at the level of the ecosystem (Chapter 10)

11.2 | Overview

While writing this thesis, the Great Barrier Reef in Australia was hit by its third and worst bleaching event since 2016[6]. Future projections are dire, since bleaching frequency will increase in response to rising sea surface temperatures due to global warming[7,8]. Reported cases of back-to-back bleaching are now widespread[9–12]. As a result, recovery times between bleaching events will shorten dramatically[13], which will likely result in a decrease in coral cover. Recent efforts to mitigate the loss of corals have led to coral restoration- and recovery-based approaches[14,15]. A recent example of such an approach has led to the development of a more thermal tolerant strain of Symbiodiniaceae[16], through assisted evolution[17]. However, these approached apply a "symptom relief" approach as they do not focus on the root cause of the problem, and it is debated whether or not these methods are feasible as long-term solutions. While the main solution is first and foremost the reduction of greenhouse gas emissions, part of the solution may be in understanding what maintains a stable coral – Symbiodiniaceae symbiosis in the first place. As such,

recent efforts to study coral reef functioning have been focusing on nutrient availability and biogeochemical cycling of these nutrients associated with a range of coral reef functional groups and the role of N-cycling microbes may be especially crucial[18–20].

The present thesis aimed to extent the current knowledge on N-cycling microbes by assessing one or two N-cycling pathways in a range of functional groups, with a strong focus on hard corals, associated with three Red Sea reefs, i.e. a northern Red Sea fringing reef (N29°27'31", E34°58'26"), and the central Red Sea reefs Abu Shosha (N22°18'15", E39°02'56") and Al Fahal (N22°15'100", E038°57'382"). All three reefs provided excellent circumstances to study biogeochemical cycling of N in response to environmental change, both natural and anthropogenically induced. To this end, we assessed the effects of environmental change, i.e. seasonality and depth, and eutrophication on biogeochemical cycling of N. While the focus was on the ecosystem engineers, i.e. the hard corals, the inclusion of other functional groups provided a more comprehensive understanding that allowed us to make inferences for coral reef functioning when exposed to global and local stressors. Our results suggest that N-cycling with each functional group is dynamically adjusted in response to one or more abiotic factors (see Figure 11.1A). Most importantly, denitrification was found to be an active pathway in coral holobionts that may be controlled by environmental N availability thereby providing evidence for the importance of this N-cycling pathway in supporting coral holobiont functioning.

11.3 | Biogeochemical cycling of N associated with functional groups in relation to coral holobionts

Coral reef organisms compete for the same scarcely available nutrients. The nutrient exchange symbiosis between corals and Symbiodiniaceae has historically given the coral an ecological advantage over its competitors. However, the consequences of climate change and local stress may give other functional groups an advantage over the corals.

The role of phytoplankton within the biogeochemical cycling of N on coral reefs has been an understudied subject. As planktonic communities investigated in Chapter 2 were net heterotrophic throughout the year and positive correlations were found between net photosynthesis and NO_x (nitrite or nitrate) and total DIN, also for N_2 fixation and NO_x/DIN (see Figure 11.1A), it is likely that the diazotroph derived N is mainly used to fuel the phytoplankton communities' own metabolism. Indeed, compared to benthic diazotrophs, N_2 fixation rates of planktonic diazotrophs were 20-fold lower[21], thus likely having a limited effect on the benthos. However, recent research suggests that bleached corals may increase heterotrophic grazing of planktonic diazotrophs[22]. As such, the results provided in Chapter 2 would suggest that the coral holobionts in the northern Red Sea may have access to a food source rich in N throughout the year if exposed to stressful conditions.

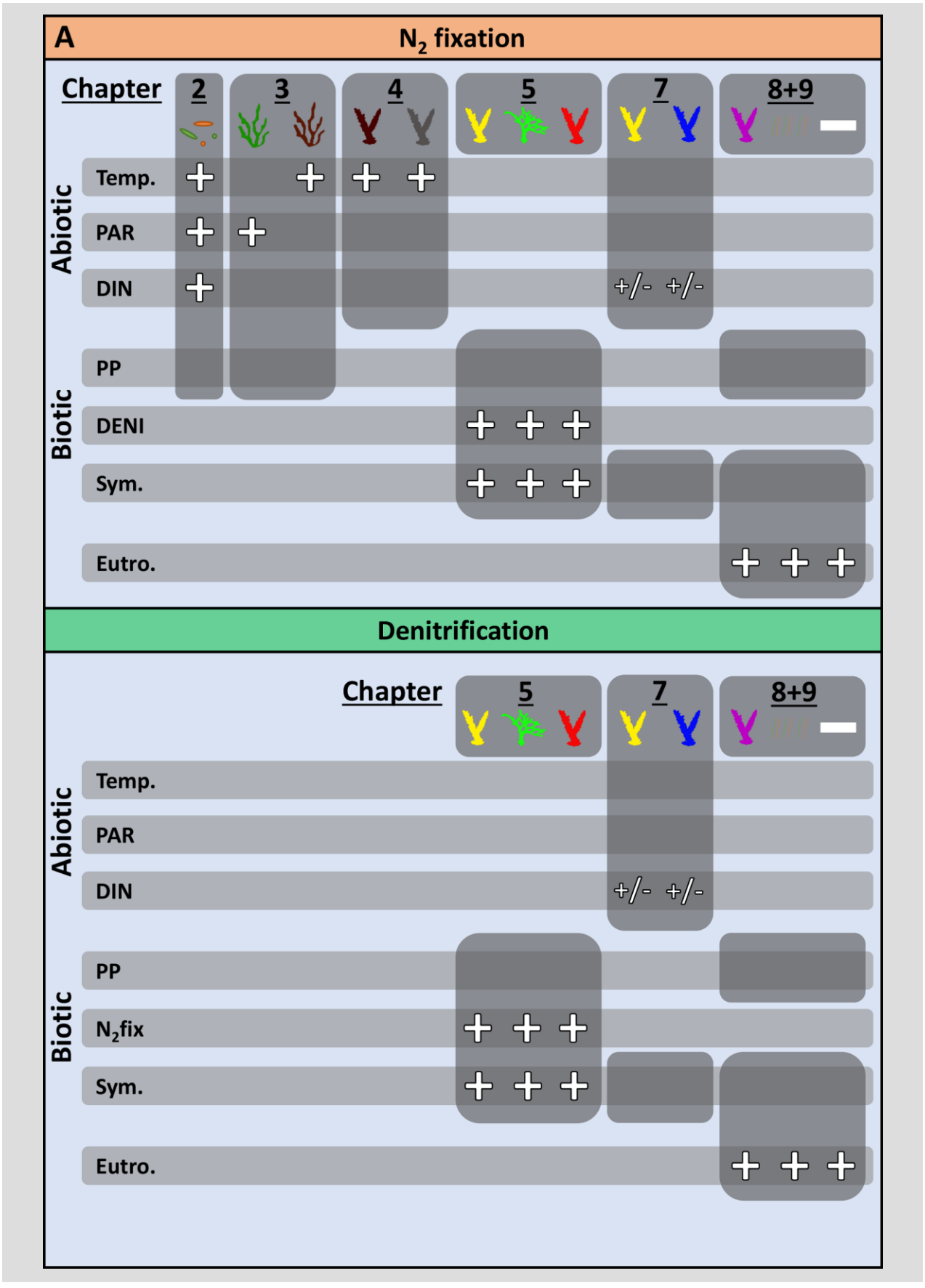
While pelagic diazotrophs may have a limited effect on the benthos, our results suggest that benthic diazotrophs associated with macroalgae are important sources of N to e.g. maintain stable primary production (Chapter 3). N_2 fixation rates measured in macroalgae were about 5-fold higher than rates measured in hard corals. As such, macroalgae may have an ecological advantage over hard corals when competing for space, especially in a high light or high water-temperature environment (see Figure 11.1A). Moreover, turf algae associated N_2 fixation rates, measured in parallel by Rix et al.[23], were about 20-fold

higher than those in hard corals. Similar results are presented in Chapter 10, thus providing additional evidence for the potentially competitive advantage of algae. In Chapter 10, we found about ~110-fold higher N_2 fixation associated with turf algae relative to hard corals and low denitrification in comparison. Accumulated, this suggests that turf algae may have an ecological advantage for occupying free space as they can quickly capitalize on anthropogenic eutrophication (Chapter 9), have high N_2 fixation rates (Chapter 3, Chapter 8, and Chapter 10) and low denitrification rates (Chapter 8 and Chapter 10). Moreover, N_2 fixation rates associated with dead coral, i.e. coral rubble, was about 95-fold higher than living hard corals. This may, in addition, explain why turf algae often overgrow dead coral at a rapid pace[24,25].

While turf algae, coral rubble, and also biogenic rock generate the most *de novo* bioavailable N through N_2 fixation, the microbial assemblages associated with these functional groups contribute the least in alleviating the benthos from bioavailable N through denitrification (Chapter 10). In fact, in both algae- and coral-dominated areas, the coral holobionts (hard and soft) contributed most to N alleviation via denitrification (Chapter 10).

11.4 | Denitrification associated with coral holobionts

Functional marker genes for denitrification were previously found in the hard corals *Montastraea faveolata*[26] (now *Orbicella faveolata*)[27], *Fungia* sp.[28], *Favia* sp.[28], *Acanthastrea* sp.[28], *Tubastraea coccinea*[29] and the soft coral species *Alcyonium gracillimum*[29] (now *Scleronephthya gracillimum*). In the current thesis we identified functional marker genes for denitrification and/or measured denitrification rates in the hard corals *Acropora hemprichii* (Chapter 5 and Chapter 7), *Pleuractis granulosa* (Chapter 5), *Stylophora pistillata* (Chapter 7 and Chapter 10), *Pocillopora verrucosa* (Chapter 8 and Chapter 10), the Hydrozoan *Millepora dichotoma* (Chapter 5), and the soft coral *Xenia* sp. (Chapter 10). As such, we can say with some confidence that denitrification is likely ubiquitously associated with most coral species. In Chapter 5 we established that the denitrification rates associated with three common Red Sea corals aligned with relative *nirS* gene copy abundances. As such, molecular quantification of the *nirS* gene can act as a proxy for denitrification potential, as applied in Chapter 7. Furthermore, we demonstrated that denitrification rates were correlated with N_2 fixation rates and Symbiodiniaceae cell densities, while N_2 fixation rates were found to correlate with denitrification rates and also with Symbiodiniaceae cell densities (see Figure 11.1A). As both denitrifiers and diazotrophs are mainly heterotrophic[30–32], the correlation between denitrification rates and N_2 fixation rates may be explained through a shared availability of organic carbon (C) from the Symbiodiniaceae. This potential interplay of denitrifiers and N_2-fixers was further investigated in Chapter 7 as we assessed the seasonal ratios of denitrifiers relative to diazotrophs. Even though the correlation with Symbiodiniaceae cell densities was not established here (see Figure 11.1A), we found evidence that both N-cycling pathways may directly support coral holobiont functioning by regulating coral holobiont N availability and by extension regulate the Symbiodiniaceae population. More evidence in support of this was found in Chapter 8 and Chapter 9. In Chapter 8 we assessed denitrification and N_2 fixation rates associated with the hard coral *Pocillopora verrucosa* in response to moderate eutrophication. Surprisingly, N_2 fixation rates were higher in the experimental corals compared to the control corals, i.e. there was no depressing effect of elevated DIN on N_2 fixation[33–35]. In response, denitrification rates also went up. Despite this two-fold N stress, i.e. increased environmental DIN and N_2 fixation, there was no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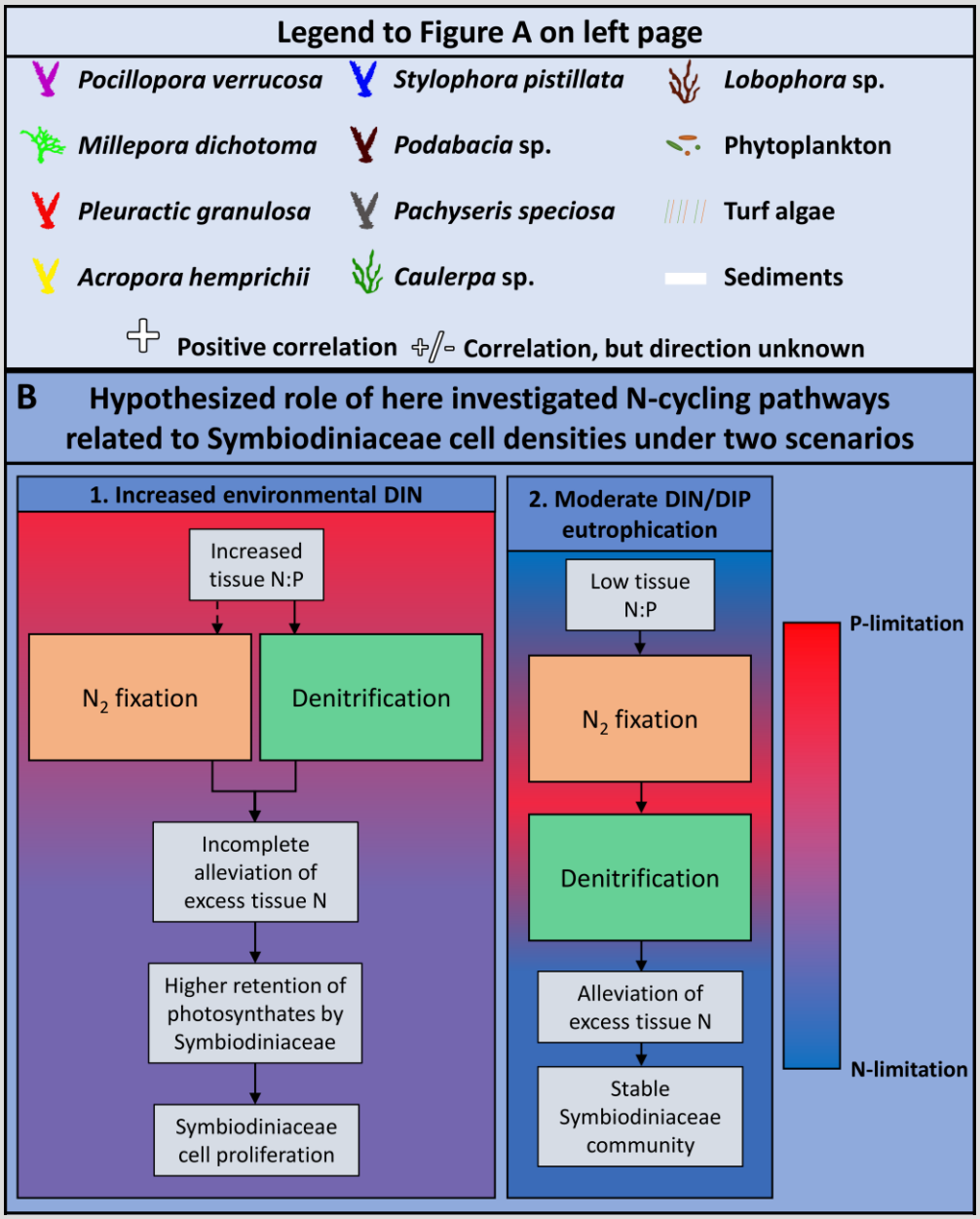

Figure 11.1 | **(A)** (left page) Overview of organisms/substrates investigated in Chapters 2-5 and 7-9 per assessed nitrogen (N) cycling pathway (N_2 fixation at the top, denitrification at the bottom) and their respective strongest biotic and/or abiotic correlations. Legend of organisms/substrates on right page (above). Temp. = water temperature, PAR = Photosynthetically Active Radiation, DIN = Dissolved Inorganic N, PP = primary production, N_2fix = N_2 fixation, DENI = denitrification, Sym. = cell densities of Symbiodiniaceae, Eutro. = Eutrophication. **(B)** Hypothesized role of N-cycling pathways, i.e. N_2 fixation and denitrification, in coral holobionts and their relation to Symbiodiniaceae cell densities under two nutrient availability scenarios. Solid arrow = positive feedback, dashed arrow = negative feedback. Figure 11.1B1 is based on results obtained in Chapter 7. As such, the direction of which the N-cycling pathways are influenced by increased environmental DIN are considered speculative (see Chapter 7). Figure 11.1B2 is based on results obtained from the eutrophication experiment described in Chapter 8 and Chapter 9. The background color of Figure 11.1B1 an Figure 11.1B2 reflects N to phosphorus (P) ratios displayed as different levels of N or P limitation in the coral holobiont.

significant increase of DIN (originating from the fertiliser) incorporated into the Symbiodiniaceae despite increased levels found in the coral tissue (Chapter 9). This may suggest that denitrifiers alleviated most of the excess N (see Figure 11.1B2), or that uptake of bioavailable N from increased N_2 fixation and the fertiliser was balanced (among other reasons, see Discussion of Chapter 9). However, higher availability of DIN may lead to increased proliferation of Symbiodiniaceae (Chapter 7), but cell densities of Symbiodiniaceae remained similar between corals from the eutrophied and control communities (Chapter 9). Though *Pocillopora verrucosa* is a hard-coral species that is considered to have a rather inflexible microbiome[36,37], cell densities of Symbiodiniaceae can still increase in response to excess N[37]. As such, I postulate that activity of denitrification is a more likely explanation for the stable Symbiodiniaceae population. In Chapter 7 we found increased cell densities of Symbiodiniaceae in both investigated corals (*Acropora hemprichii* and *Stylophora pistillata*) during the fall, likely in response to high nitrate availability. Thus, we hypothesize that the dynamic interaction of N_2 fixers and denitrifiers was not optimal to fully alleviate excess N (see Figure 11.1B1). Even though DIN availability during the fall was in a similar range as DIN availability during the eutrophication experiment, the latter also included phosphorus (P), despite not being measured within the experimental communities, possibly due to rapid uptake. Indeed, P may positively affect denitrification[38]. Thus, either the ratio of DIN to dissolved inorganic P (DIP) ratios may be an important factor controlling N_2 fixation / denitrification dynamics or the extent to which denitrification may alleviate N from the coral holobiont is species specific.

11.5 | Linking coral holobiont denitrification to trophic strategy

The correlation between denitrification, N_2 fixation and Symbiodiniaceae cell densities found in Chapter 5 led us to speculate that translocated photosynthates from the Symbiodiniaceae may be the main energy source for both N-cycling pathways. As such, corals that rely heavily on the photosynthates translocated by the Symbiodiniaceae, i.e. are on the autotrophic end of the mixotrophic spectrum, may rely more on denitrification and N_2 fixation than corals on the heterotrophic end of the mixotrophic spectrum. Supporting evidence for this hypothesis pertaining to N_2 fixation was first provided by Pogoreutz et al.[39]. In Chapter 4 we present further evidence in support of this hypothesis. The symbiotic relationship with diazotrophs may be beneficial for coral holobiont under stable conditions[40] in shallow water, but may be detrimental to coral holobiont functioning in response to stressful environmental change[19,41]. Indeed, corals with higher heterotrophic capacity (defined here as: the ability of the coral holobiont to utilize organic C from heterotrophic feeding in the absence or reduced availability of photosynthates from autotrophy) may have a lower susceptibility not just to eutrophication[42], but also to increasing sea surface temperatures[43,44]. Due to the potential similarities between diazotrophy and denitrification, e.g. both being heterotrophic facultative anaerobes, it is tempting to speculate that denitrification may be a more active pathway in coral holobionts with low heterotrophic capacity as the denitrifiers may rely on photosynthates released by the Symbiodiniaceae, thus depending on the dietary mode of the coral holobiont (visualized in Figure 11.2). If true, this could have severe implications for coral functioning under environmental change.

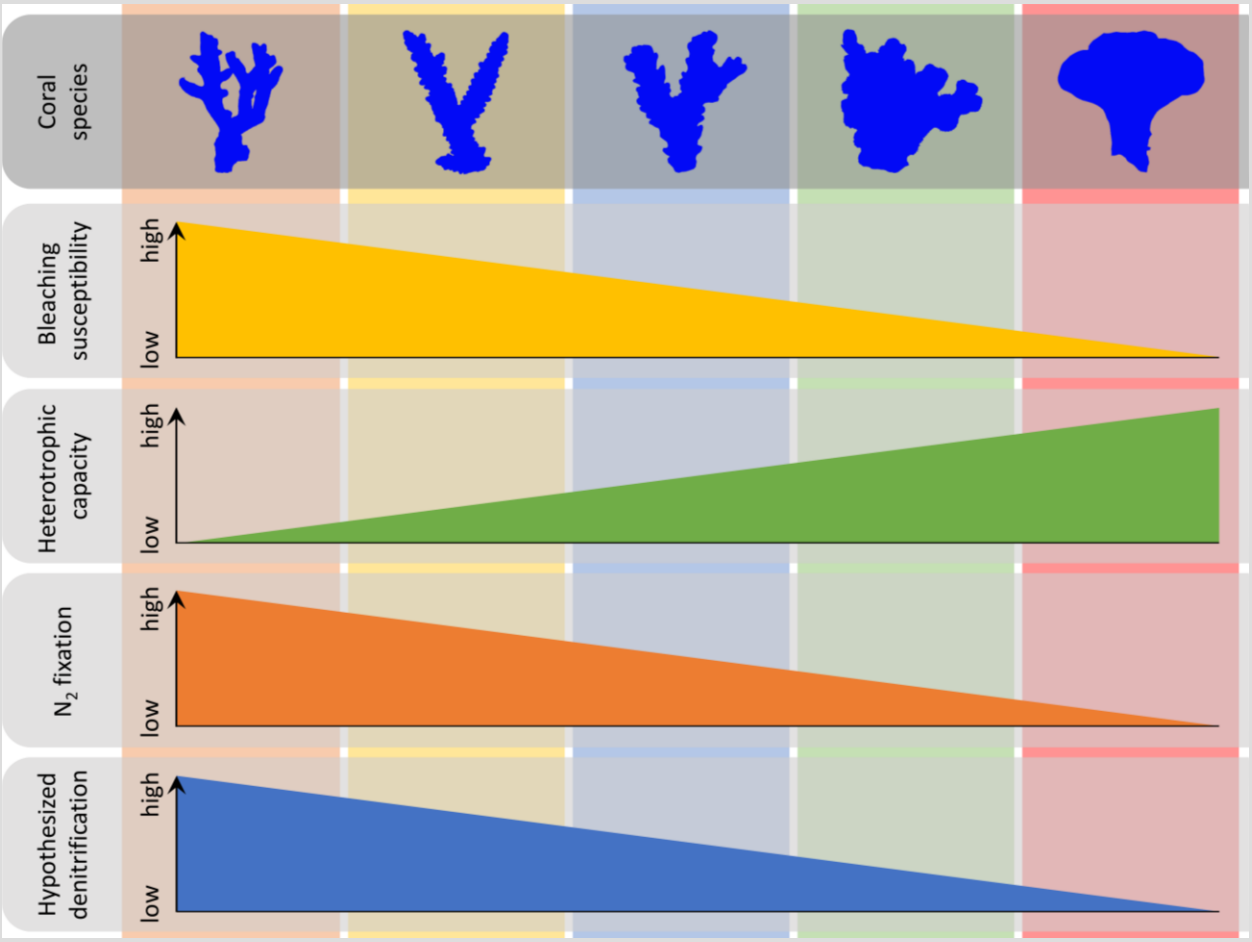

Figure 11.2 | Conceptual design of hypothesized physiological characteristics in coral holobionts. Coral species with low heterotrophic capacity are hypothesized to have high denitrification activity (Chapter 5, 7 and 8). Outlines of coral holobionts are shown for illustrative purposes only to represent five different species with increasing heterotrophic capacity. Figure design courtesy of N. Rädecker.

While the heterotrophic capacity of coral species used in this thesis was not experimentally established, based on the available literature the coral species were assigned to one of five possible trophic strategies (Figure 11.3):

1) Fully autotrophic: Coral species that rely solely on autotrophy
2) Autotrophic mixotrophic: Coral species that are mixotrophic but are more on the autotrophic end of the mixotrophic spectrum
3) Mixotrophic: Coral species that rely on a more balanced mix of autotrophy and heterotrophy
4) Heterotrophic mixotrophic: Coral species that are mixotrophic but are more on the heterotrophic end of the mixotrophic spectrum
5) Fully heterotrophic: Coral species that rely solely on heterotrophy, i.e. azooxanthellate corals

Acropora hemprichii, used in Chapter 5, is a mixotrophic coral at the autotrophic end of the mixotrophic spectrum[45], *Millepora dichotoma* is likely mixotrophic[46], while *Pleuractis granulosa* is more on the heterotrophic end[47] of the mixotrophic spectrum (Figure 11.3). Following the above described hypothesis, from herein referred to as the denitrification-heterotrophic capacity hypothesis (DHC hypothesis), denitrification rates should be highest in *A. hemprichii* and lowest in *P. granulosa*, while somewhere in between for *M. dichotoma*. Indeed, the pattern of denitrification rates (Figure 5.2) align with the estimated trophic strategy of the coral species. While this is limited evidence to confirm the DHC hypothesis, additional evidence is provided in Chapter 10. As denitrification may be prone to seasonal differences (see Chapter 7), denitrification rates cannot be directly compared between coral species used in both Chapter 5 and Chapter 10 as they were not sampled simultaneously. However, in Chapter 10 we found that denitrification rates associated with *Xenia* sp. were about 6-fold higher than the three investigated hard corals (*A. hemprichii*, *P. verrucosa*, and *S. pistillata*). Indeed, *Xenia* sp. are considered functional autotrophs[48,49] and thus have a lower heterotrophic capacity compared to the hard corals (Figure 11.3). Furthermore, the reliance on autotrophy coupled with relative high importance of N-cycling microbes may thus explain the high bleaching susceptibility of *Xenia*[49] (Figure 11.2 and Figure 11.3).

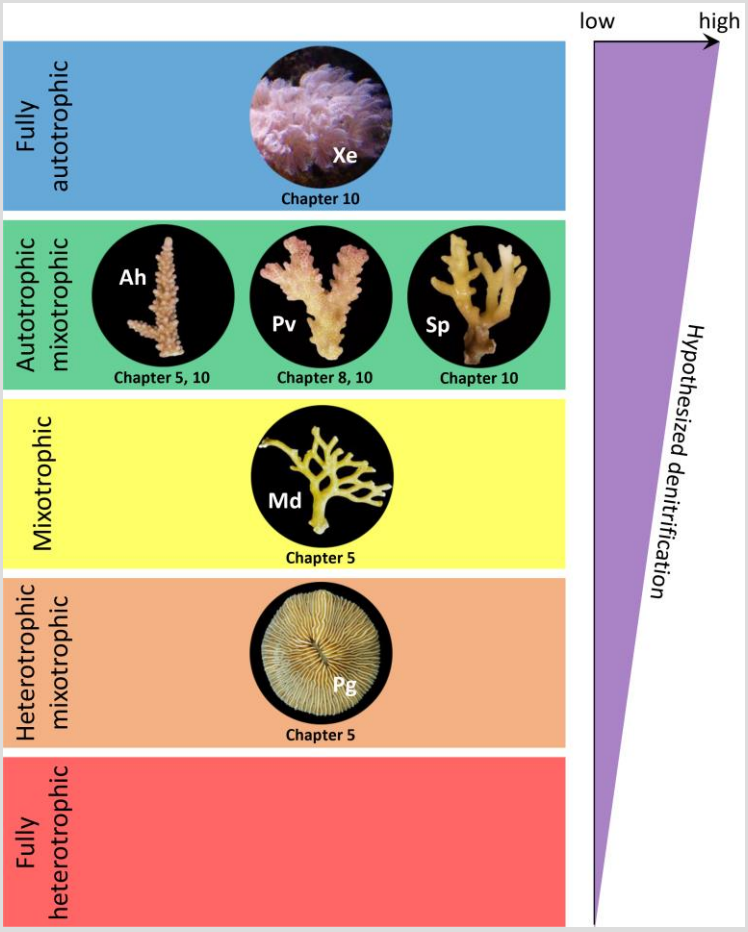

Figure 11.3 | Estimated heterotrophic capacity of the coral species used in this thesis and hypothesized relative denitrification rates (see text). Xe = *Xenia* sp., Ah = *Acropora hemprichii*, Pv = *Pocillopora verrucosa*, Sp = *Stylophora pistillata*, Md = *Millepora dichotoma*, Pg = *Pleuractis granulosa*. Photographs by Arjen Tilstra.

11.6 | Synoptic answers to the specific research questions

Research question 1: Is N_2 fixation associated with coral reef organisms affected by environmental change and how do these potential changes compare between organisms? What are the ecological implications?

Temperature was the abiotic variable that correlated strongest with 4 of the 5 organisms (Chapter 2; Chapter 3; Chapter 4; Figure 11.1A). However, correlations with PAR and DIN where also found for phytoplankton (depending on the season in which the organisms were sampled and whether seasons were pooled) and one of the macroalgae. All three correlations are commonly known to alter N_2 fixation rates[23,50–53]. The correlation with water temperature is especially important as this may have strong implications for the functioning of these organisms under global warming scenarios. Whereas phytoplankton and macroalgae may benefit from increased water temperatures and subsequent increased activity of diazotrophs, for coral holobionts this may be detrimental[19]. As such, both phytoplankton and macroalgae may have an ecological advantage, compared to coral holobionts, when exposed to higher water temperatures.

Research question 2: Is denitrification an active pathway associated with coral holobionts? If yes, how does it align with abiotic and/or other coral holobiont associated biotic variables?

Our results suggest that denitrification is indeed an active N-cycling pathway in coral holobionts (Chapter 5). Furthermore, it was found to align with N_2 fixation and density of the Symbiodiniaceae associated with the respective coral species (Figure 11.1A). In addition, we demonstrated that the relative abundances of the functional marker gene *nirS* can be used as a proxy for denitrification rates. By assessing denitrification in relation to diazotrophy in Chapter 7 we were able to find evidence that the ratio between these two pathways may be dynamically controlled by environmental DIN availability and thus act as a buffer against seasonal changes in environmental DIN availability (to a certain extent). Thereby, the interplay of denitrifiers and diazotrophs may directly assist the coral host in regulating Symbiodiniaceae populations (Figure 11.1B).

Research question 3: How are N-cycling pathways associated with several functional groups affected by eutrophication? Do these functional groups incorporate the extra bioavailable N?

Eutrophication increased N_2 fixation and denitrification rates associated with carbonate sediments, turf algae and a hard coral (Chapter 8). This resulted in increased oxygen evolution in sediments, but not for turf algae and hard corals. While fertilizer N was incorporated in the hard corals' tissue, no significant uptake was reported for the Symbiodiniaceae (Chapter 9). Furthermore, Symbiodiniaceae cell densities of the hard corals remained comparable between the control and eutrophied communities (Chapter 9). As such, fertilizer N may have been effectively alleviated via denitrification (Figure 11.1B2). Soft corals, of which N_2 fixation and denitrification rates were not tested, and especially turf algae capitalized most on fertiliser N, as high incorporation was found in both functional groups.

Research question 4: Which functional groups are the major contributors to N-cycling on coral reefs? Do differences in benthos composition affect the cycling of N and what are the ecological implications?

Turf algae, coral rubble, hard corals and soft corals are major contributors within the biogeochemical cycling of N (Chapter 10). However, the direction to which they contribute is different. Whereas turf algae and coral rubble fix the most N, both in a coral- and an algae-dominated reef, their contribution to alleviation of N is relatively low. Contrastingly, corals, both hard and soft, contribute little to the influx of N through N_2 fixation in coral- and algae-dominated reefs, but they contribute the most when it comes to alleviating the reefs from bioavailable N. As such, coral reefs in the process of shifting towards algae dominance may get caught in a positive feedback loop where dead coral is rapidly overgrown by algae which in return naturally provide the reef with bioavailable N thereby likely facilitating higher growth rates of the algae.

11.7 | Perspective and future research directions

This thesis adds to a growing body of knowledge highlighting the potential functional importance of biogeochemical cycling of N by coral reef associated N-cycling microbes, the role of denitrifiers in particular. This thesis provides the first evidence that denitrification is an active N-cycling pathway in coral holobionts. Coral holobionts may not only alleviate most bioavailable N through denitrification at the ecosystem level, this N-cycling pathway may be of functional importance at the individual level. In this thesis I hypothesize the role of coral holobiont associated denitrifiers in two different DIN:DIP ratio scenarios:

1) High availability of N, i.e. high tissue DIN:DIP ratios, may result in the proliferation of Symbiodiniaceae due to incomplete alleviation of N via denitrification (Figure 11.1B1).
2) Moderate eutrophication of both DIN and DIP, resulting in low tissue DIN:DIP ratios, may result in maintaining stable Symbiodiniaceae communities due to full alleviation of excess N via denitrification (Figure 11.1B2).

If correct, amending reefs with P may be a short-term solution in times of N eutrophication or temperature induced stimulation of diazotrophic activity to maintain the coral holobionts internal N:P ratio in a suitable range. However, adverse effects of P fertilization are unknown and warrants further investigation.

While this thesis provides the first evidence for the functional significance of denitrification in coral holobionts, the functional role of denitrification associated with coral holobionts still remains speculative at this point. Thus, the direction of future research on denitrification associated with coral holobionts has been highlighted in the Discussions of Chapter 5 and Chapter 7. In addition, Chapter 8 and Chapter 10 discusses ecosystem-level open questions, pertaining to denitrification and its potential interaction with N_2 fixation, that deserve more attention. In turn, Chapter 10 answers one question raised in Chapter 8.

In summary, future research should focus on:

a) locating the main N-cycling microbial players in coral holobionts, including those capable of nitrification and ANAMMOX (if present), along with an accurate quantification of metabolic interactions with other holobiont members.
b) studying the effects of anthropogenically–driven change on N-cycling microbes on the coral holobiont and ecosystem level.
c) detecting and quantifying the interaction between N-cycling pathways described in this thesis with other possibly ubiquitous N-cycling pathways in coral reef ecosystems.

In addition, the hypothesis that denitrification may be linked to the heterotrophic capacity of the coral holobiont (DHC hypothesis: described on pages 191-193 and visualized in Figure 11.2) deserves more attention. For instance, the quantification of denitrification rates associated with fully heterotrophic holobionts are lacking to date, despite denitrifiers being present in at least two azooxanthellate corals[29]. Heterotrophic capacity can be established several ways (see for instance [45,54,55]) and can then be aligned with denitrification rates, activity and/or to relative/absolute abundances of functional marker genes involved in the denitrification pathway. Concerning the latter, knowledge from molecular ecology on denitrification in coral holobionts currently only pertains to nitrite reductase (*nir*) containing denitrifiers[29] (Chapter 5 and Chapter 7). Thus, understanding the dynamics of denitrification pertaining to other parts of the denitrification pathway in coral holobionts, i.e. nitrate reductase (gene cluster *nar*), nitric oxide reductase (gene cluster *nor*), and nitrous oxide reductase (gene cluster *nos*)[56,57], remains yet to be determined and is pending specific primer design[4].

11.8 | References

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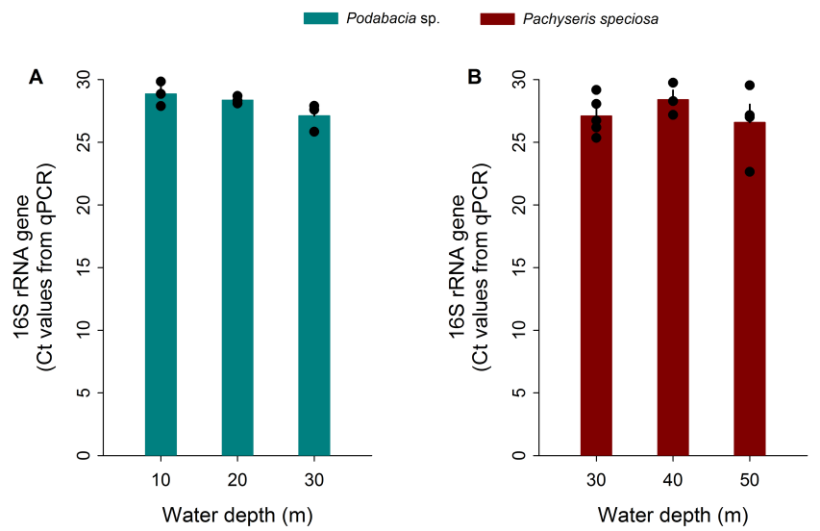
Appendix

Appendix: Supplementary material

Supplementary material to Chapter 4

Supplementary Figures



Supplementary Figure S4.1 | Ct values from quantitative PCR of 16S rRNA gene amplicons at three different water depths for **(A)** *Podabacia* sp. and **(B)** *Pachyseris speciosa*. Black circles represent data points.

BEST
Biota and/or Environment matching

Data worksheet
Name: Env_Par_Norm
Data type: Environmental
Sample selection: All
Variable selection: All

Resemblance worksheet
Name: Bio_Rep_Resem
Data type: Similarity
Selection: All

Parameters
Rank correlation method: Spearman
Method: BIOENV
Maximum number of variables: 1
Resemblance:
Analyse between: Samples
Resemblance measure: D1 Euclidean distance

Variables
1 Temperature
2 PAR
3 Oxygen
4 Chlorophyll

Global Test
Sample statistic (Rho): 0.317
Significance level of sample statistic: 0.3%
Number of permutations: 999 (Random sample)
Number of permuted statistics greater than or equal to Rho: 2

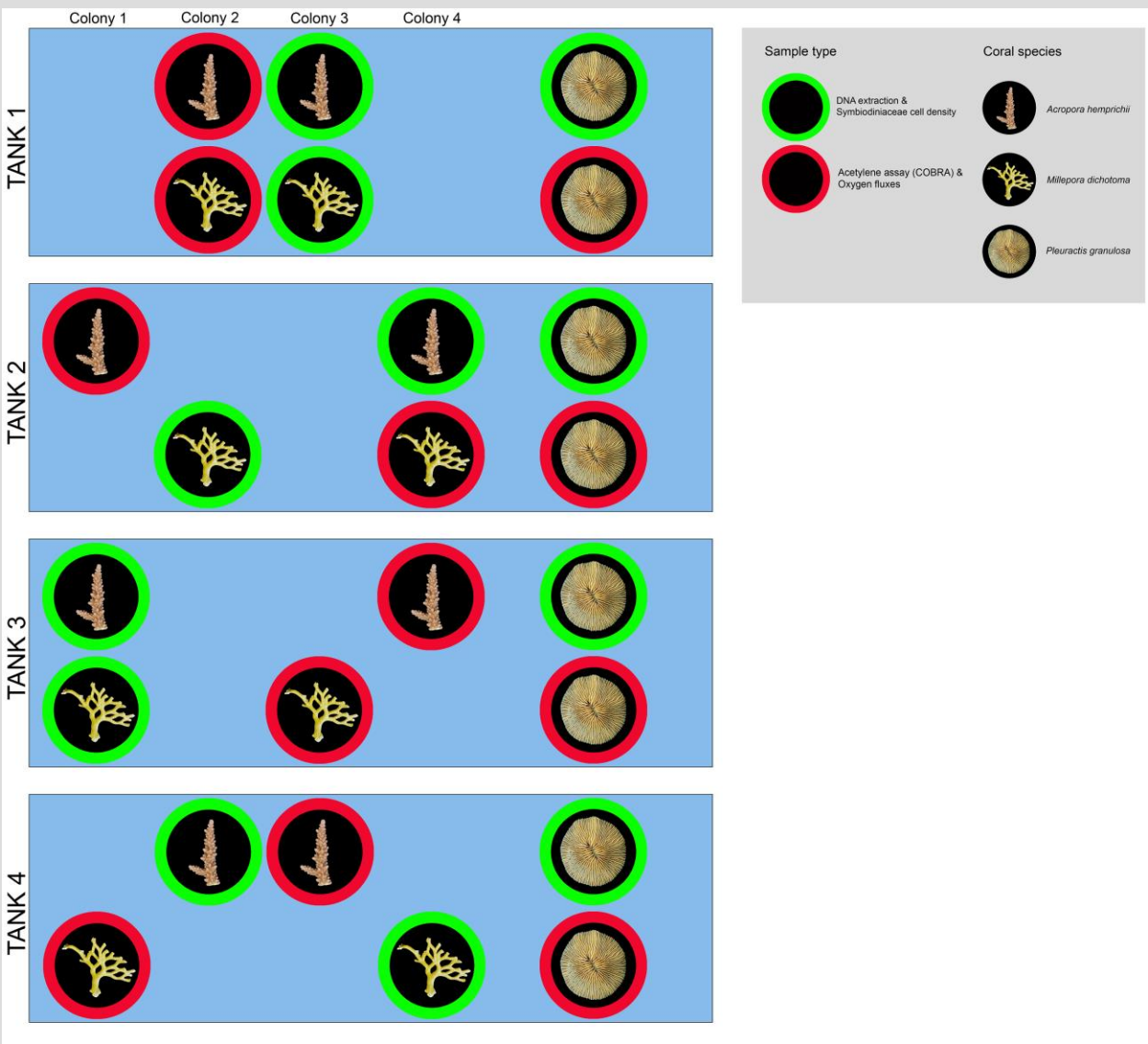
Best results

No.Vars	Corr.	Selections
1	0.317	1
1	0.289	4
1	0.269	2
1	0.105	3

Supplementary Figure S4.2 | Results of BIOENV analysis performed with PRIMER 6.

Supplementary material to Chapter 5

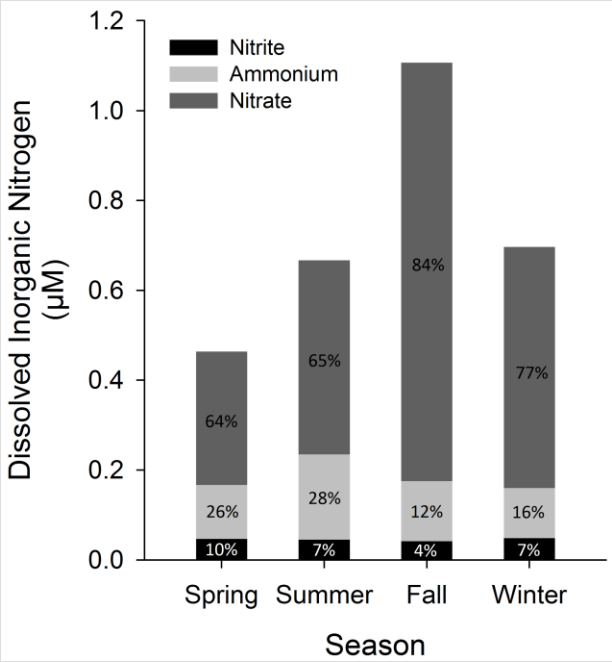
Supplementary Figures



Supplementary Figure S5.1 | Overview of sampling scheme of fragmented coral colonies, i.e. four colonies of *A. hemprichii*, four colonies of *M. dichotoma* and eight individual polyps of *P. granulosa*, which were divided over four independent replicate tanks. The outline colour denotes the type of analysis that the sampled fragments were used for.

Supplementary material to Chapter 7

Supplementary Figures



Supplementary Figure S7.1 | Proportions of dissolved nitrogenous compounds. Data taken from Figure 7.2. Numbers in the bars represent the total percentage of the nitrogenous compound pertaining to total DIN per season.

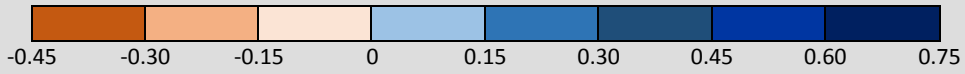
Supplementary Tables

Supplementary Table S7.1 | Summary of significant main effects and interactions based on permutational multivariate analysis of variance (PERMANOVA) for *nirS to nifH* gene ratios and Symbiodiniaceae cell densities.

nirS to nifH gene ratios			
	df	Pseudo-F	*p*
Season	3	3.04	0.039
Species	1	6.11	0.019
Season x Species	2	0.14	0.877
Symbiodiniaceae cell densities			
	df	Pseudo-F	*p*
Season	3	10.16	0.001
Species	1	0.52	0.473
Season x Species	3	3.19	0.037

Supplementary Table S7.2 | Correlation coefficients for *nirS* to *nifH* gene ratios and Symbiodiniaceae cell density with eight environmental parameters (based on Pearson Product-Moment Correlation analysis).

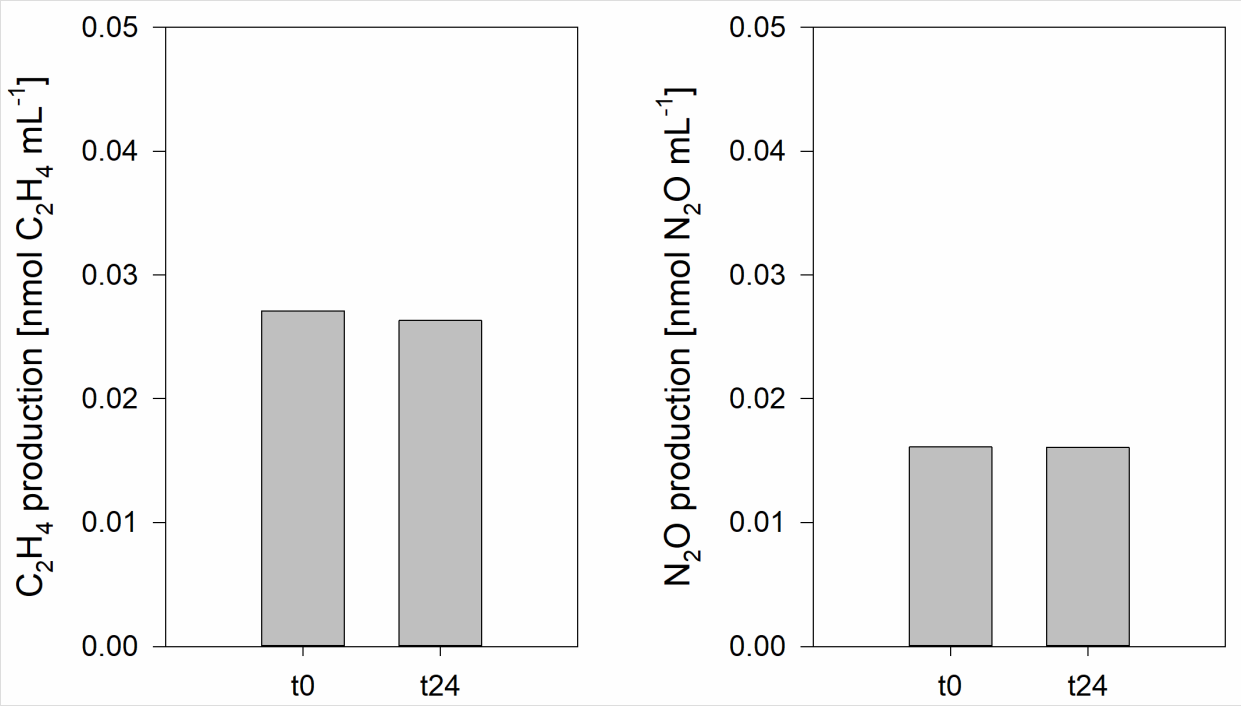
	Temp.	PAR	DO	NO_3^-	NH_4^+	DIN	DIP	DOC
nirS to *nifH* gene ratios				**		*		
Symbiodiniaceae								
A. hemprichii	*			***		***		*
S. pistillata				*		*		



Temp. = temperature, PAR = photosynthetically active radiation, DO = dissolved oxygen, NO_3^- = nitrate, NH_4^+ = ammonium, DIN = dissolved inorganic nitrogen, DIP = dissolved inorganic phosphorus, DOC = dissolved organic carbon. The correlation coefficient is indicated by the colour chart below the table. Asterisks indicate significant correlations. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Supplementary material to Chapter 8

Supplementary Figures



Supplementary Figure S8.1 | Ethylene (C_2H_4; left) and nitrous oxide (N_2O; right) production in control incubation chambers (n = 2; with 5 µM nitrate addition) over the whole incubation time of 24 h with samplings at the beginning (t_0) and the end (t_{24}) of incubations.

Supplementary Tables

Supplementary Table S8.1 | Osmocote ® Fertiliser composition

Coated Micronutrient	Amount
Ammoniacal Nitrogen*	8 %
Nitrate*	7 %
Phosphate (P_2O_5)*	9 %
Soluble Potash (K_2O)*	12 %
Magnesium*	1.3 %
Sulfur*	5.9 %
Boron	0.02 %
Copper	0.05 %
Iron*	0.46 %
Manganese*	0.06 %
Molybdenum*	0.02 %
Zinc	0.05 %

* = micronutrients have been coated to provide 14.77 % coated slow-release nitrogen, 8.86 % coated slow-release available phosphate (P_2O_5), 11.81 % coated slow-release soluble potash (K_2O), 1.27 % coated slow-release magnesium, 5.85 % coated slow-release sulphur, 0.45 % coated slow-release iron, 0.059 % coated slow-release manganese, and 0.019 % coated slow-release molybdenum.

Supplementary Table S8.2 | Differences (given as p-values*) in gross primary production (P_g), N_2 fixation and denitrification between functional groups of control and eutrophied communities.

	P_g	N_2 Fixation	Denitrification
Reef Sediment	**0.002**	**0.001**	0.081
Turf Algae	0.163	0.095	**0.003**
Pocillopora verrucosa	0.636	0.338	0.140

* = obtained via two-way permutational multivariate analysis of variance (PERMANOVA) with the factors "functional group" and "community" (i.e., control and eutrophied communities). Significant *p*-values in bold.

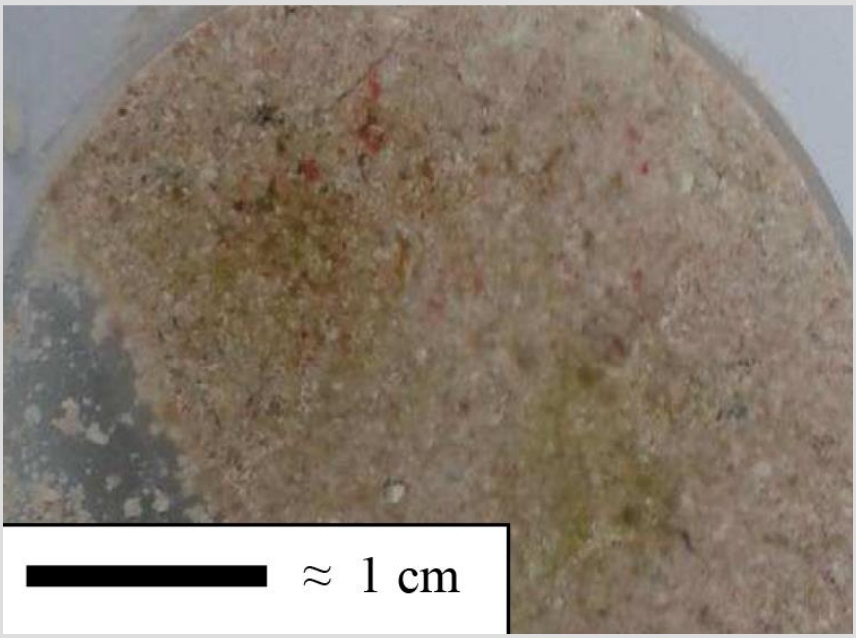
Supplementary Table S8.3 | Results (*p*-values) of permutational multivariate analysis of variance (PERMANOVA) pair-wise tests for N_2 fixation and denitrification in all functional groups of control (Ctrl., blue) and eutrophied (Eut, green) communities.

		Control		Eutrophied			
		Turf Algae	*P. verrucosa*	Reef sediment	Turf Algae	*P. verrucosa*	
Ctrl	Reef Sediment	**0.001**	**0.001**	**0.001**	**0.001**	**0.004**	N_2 fixation
	Turf Algae		**0.001**	**0.019**	0.095	**0.001**	
	P. verrucosa			**0.001**	**0.001**	0.338	
Eut	Reef Sediment				**0.017**	**0.001**	
	Turf Algae					**0.001**	
Ctrl	Reef Sediment	0.536	0.170	0.081	0.068	0.923	Denitrification
	Turf Algae		0.103	**0.017**	**0.003**	0.473	
	P. verrucosa			**0.006**	**0.001**	0.140	
Eut	Reef Sediment				0.587	0.122	
	Turf Algae					0.098	

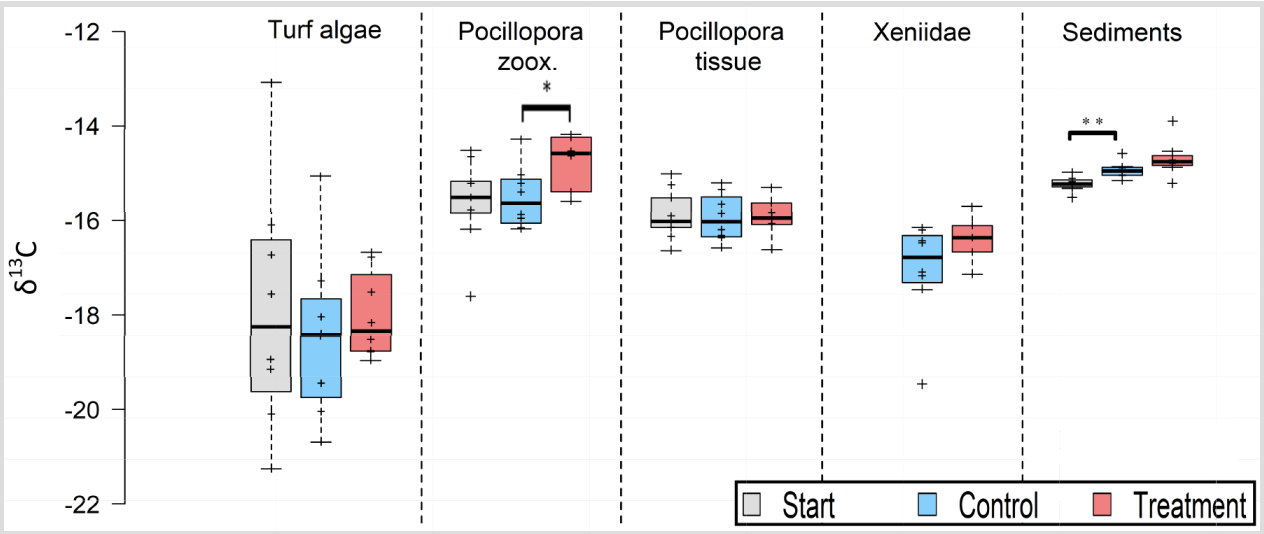
Significant *p*-values in bold.

Supplementary material to Chapter 9

Supplementary Figures



Supplementary Figure S9.1 | Sediment sample with epilithic algae after 8 weeks of *in situ* eutrophication.



Supplementary Figure S9.2 | $\delta^{13}C$ in turf algae, *Pocillopora* cf. *verrucosa* zooxanthellae ("zoox.") and tissue, Xeniidae and sediments before (grey), without (blue) and after 8 weeks in-situ eutrophication (red). Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.005$).

Supplementary Tables

Supplementary Table S9.1 | Nutrients released by the Osmocote® fertilizer bags around manipulated reef communities measured at different distances.

Location	Time reference	Temp (°C)	Distance from fertilizer (cm)	NO_2^- (µM)	PO_4^{3-} (µM)	NO_3^- (µM)	NH_4^+ (µM)	DIN (µM N)	DIN/ PO_4^{3-}
Background*	Start	24.90	---	0.04	0.06	0.34	0.13	0.51	8.37
Centre	2 weeks		25	0.02	0.06	0.91	0.20	1.14	22.37
Fertilizer	2 weeks		0	0.03	0.44	5.68	1.34	7.06	17.00
Background	2 weeks	26.27	200	0.01	0.09	0.27	0.05	0.33	3.53
Centre	4 weeks		25	0.06	0.12	1.07	0.24	1.37	11.68
Fertilizer	4 weeks		0	0.06	0.57	5.52	1.66	7.24	13.99
Background	4 weeks	26.48	200	0.07	0.15	0.33	0.09	0.49	3.36
Centre	6.5 weeks		25	0.03	0.20	1.16	0.21	1.41	9.22
Fertilizer	6.5 weeks		0	0.05	0.30	5.04	1.49	6.59	40.79
Background	6.5 weeks	27.34	200	0.03	0.06	0.09	0.09	0.20	3.21
Background*	8 weeks	28.04	---	0.05	0.07	0.36	0.16	0.56	13.47

Fertilizer: directly at the pin, Centre: 25 cm towards the communities. Temp. = temperature, NO_2^- = nitrite, PO_4^{3-} = phosphate, NO_3^- = nitrate, NH_4^+ = ammonium, DIN = dissolved inorganic nitrogen. DIN was calculated as $[NO_2^-] + [NO_3^-] + [NH_4^+]$. *Indicates measurements from the reef water column.

Supplementary Table S9.2 | Osmocote® fertilizer elemental and stable isotope composition.

	$\delta^{15}N$	% N	$\delta^{13}C$	% C_{org}	C_{org}/N
Mean	16.326	7.722	-27.027	6.166	0.800
SD	0.513	0.416	0.859	0.328	0.046

SD indicates the standard deviation (n = 4). N = nitrogen, C = carbon, C_{org} = organic carbon.

Supplementary material to Chapter 10

Supplementary Tables

Supplementary Table S10.1 | Description of incubated functional groups, and respective 2D:3D conversion factor.

Functional Group	2D:3D*	Description/incubated organism or substrate
Hard Corals	7.6	*Pocillopora, Acropora, Stylophora*
Soft Corals	6.5	Xeniidae
Biogenic Rock	2.5	Biogenic reef framework with visible carbonate structure[1]
Coral Rubble	5.2	Equal to coral rock, uncovered dead coral skeleton[2]
Turf Algae	3.9	Dense mats composed of a heterogeneous assemblage of filamentous algae
Reef Sands	1.0	Surface layer with its associated microphytobenthos

*According to Cardini et al.[3]

Supplementary Table S10.2 | Relative benthic cover in coral- and algae-dominated reef areas.

Functional Group	Relative cover [%] in coral-dominated area	Relative cover [%] in algae-dominated area
Hard Coral	43.55 ± 5.67	13.96 ± 2.33
Soft Coral	8.85 ± 3.78	8.13 ± 1.68
Turf Algae	26.15 ± 5.02	47.50 ± 7.15
Biogenic Rock	16.11 ± 3.05	1.25 ± 0.71
Coral Rubble	3.30 ± 3.11	17.08 ± 5.75
Reef Sands	0.52 ± 0.27	11.46 ± 3.66
Tridacna	1.34 ± 0.82	0.00 ± 0.00
Macro Algae	0.17 ± 0.00	0.63 ± 0.32
Sponges	0.00 ± 0.00	0.00 ± 0.00

Presented are mean proportional cover in % ± standard error of major benthic functional groups assessed by photo quadrats (n = 10 in coral-dominated areas, n = 12 in algae-dominated areas).

Supplementary Table S10.3 | Dinitrogen (N_2) fixation and denitrification rates of investigated functional groups.

Functional Group	Replicates	N_2-fixation	Denitrification
Turf Algae	5	570.30 ± 59.18	6.01 ± 1.10
Coral Rubble	4	482.07 ± 88.40	10.57 ± 1.99
Soft Coral	5	14.62 ± 4.16	36.79 ± 9.02
Biogenic Rock	5	97.77 ± 15.99	10.64 ± 2.44
Reef Sands	5	45.69 ± 6.07	11.25 ± 3.01
Hard Corals	13	5.11 ± 1.89	6.47 ± 2.27
Pocillopora verrucosa	5	2.52 ± 0.42	3.71 ±0.77
Acropora hemprichii	4	6.57 ± 3.23	15.80 ± 4.56
Stylophora pistillata	4	6.88 ± 4.86	0.59 ± 0.25

Rates are presented in mean ± standard errors and in pmol N_2 cm^{-2} h^{-1}. Data for hard corals consists of mean values of *P. verrucosa*, *A. hemprichii* and *S. pistillata*.

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Other publications during the candidature

Light induced intraspecific variability in response to thermal stress in the hard coral *Stylophora pistillata*

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Abstract

Recent research suggests that prior exposure of several months to elevated irradiance induces enhanced thermal tolerance in scleractinian corals. While this tolerance has been reported at the species level, individual coral colonies may react differently due to individual variability in thermal tolerance. As thermal anomalies are predicted to become common in the upcoming future, intraspecific variation may be key to the survival of coral populations. In order to study light−history based thermal stress responses on individual colonies, we developed a preliminary microcosm experiment where three randomly chosen, aquacultured colonies of the model coral *Stylophora pistillata* were exposed to two irradiance treatments (200 and 400 µmol photons m^{-2} s^{-1}) for 31 days, followed by artificially induced heat stress (~33.4 °C). We found different responses to occur at both the intraspecific and the intracolonial levels, as indicated by either equal, less severe, delayed, and/or even non−necrotic responses of corals previously exposed to the irradiance of 400 compared to 200 µmol photons m^{-2} s^{-1}. In addition, all individual colonies revealed light−enhanced calcification. Finally, elevated irradiance resulted in a lower chlorophyll *a* concentration in one colony compared to the control treatment, and the same colony displayed more rapid bleaching compared to the other ones. Taken together, this study highlights the potential importance of intra−individual variability in physiological responses of scleractinian corals and provides recommendations for improving methodological designs for future studies.

Tilstra, A., Wijgerde T., Dini−Andreote F., Eriksson B.K., Salles J.F., Pen I., Osinga R., Wild C. Light induced intraspecific variability in response to thermal stress in the hard coral *Stylophora pistillata*. *PeerJ* **5**, e3802 (2017). https://doi.org/10.7717/peerj.3802

A meta-analysis to assess long-term spatiotemporal changes of benthic coral and macroalgae cover in the Mexican Caribbean

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Abstract

Coral reefs in the wider Caribbean declined in hard coral cover by ~80 % since the 1970s, but spatiotemporal analyses for sub-regions are lacking. Here, we explored benthic change patterns in the Mexican Caribbean reefs through meta-analysis between 1978 and 2016 including 125 coral reef sites. Findings revealed that hard coral cover decreased from ~26 % in the 1970s to 16 % in 2016, whereas macroalgae cover increased to ~30 % in 2016. Both groups showed high spatiotemporal variability. Hard coral cover declined in total by 12 % from 1978 to 2004 but increased again by 5 % between 2005 and 2016 indicating some coral recovery after the 2005 mass bleaching event and hurricane impacts. In 2016, more than 80 % of studied reefs were dominated by macroalgae, while only 15 % were dominated by hard corals. This stands in contrast to 1978 when all reef sites surveyed were dominated by hard corals. This study is among the first within the Caribbean region that reports local recovery in coral cover in the Caribbean, while other Caribbean reefs have failed to recover. Most Mexican Caribbean coral reefs are now no longer dominated by hard corals. In order to prevent further reef degradation, viable and reliable conservation alternatives are required.

Contreras-Silva, A.I., **Tilstra, A.**, Migani, V., Thiel, A., Pérez-Cervantes, E., Estrada-Saldivar, N., Elias, X., Mott, C., Alvarez-Filip, L., Wild, C. A meta-analysis to assess long-term spatiotemporal changes of benthic coral and macroalgae cover in the Mexican caribbean. *Sci. Rep.* **10**, 8897 (2020). https://doi.org/10.1038/s41598-020-65801-8

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