Effects of Anthropogenic Changes on Nitrogen Cycling Microbes of Coral Holobionts

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Summary

Corals have co-evolved in symbiotic relationships with diverse eukaryotic and prokaryotic microbes, collectively termed "the coral holobiont". The efficient nutrient exchange between the coral host and photosynthetic Symbiodiniaceae supports the high productivity of corals in oligotrophic waters, yet critically depends on nitrogen limitation within the holobiont. Nitrogen cycling prokaryotes (bacteria and archaea) are thus key players in the functioning of coral holobionts. Octocorals are important benthic reef organisms, yet little is known about their-associated nitrogen cycling prokaryotes under environmental change. Denitrifiers (i.e., prokaryotes capable of reducing nitrate/nitrite to N_2) could help maintain nitrogen limitation in holobionts, yet their community structure and (a)biotic controls are still unknown. This thesis aims to extend the current knowledge of nitrogen cycling prokaryotes in coral holobionts by focusing on the following research objectives: 1) investigating the effects of dissolved organic carbon (DOC) and concomitant heat stress on the abundance of diazotrophs (i.e., prokaryotes capable of converting N_2 into ammonia) and denitrifiers in two octocoral species with distinct trophic strategies: the more mixotrophic soft coral *Xenia umbellata* and the highly autotrophic gorgonian *Pinnigorgia flava*; 2) investigating the respective contributions of host and algal identities to the structuring of denitrifier communities in cnidarian holobionts; 3) characterizing denitrifier communities in cnidarian holobionts and identifying their driving factors. The experimental work consisted of a multidisciplinary approach combing physiological, molecular, and bioinformatical tools and executed over the course of two aquarium-based experiments: 1) a 45-day experiment consisting of 21 days of DOC enrichment at ambient 26 ^{o}C followed by 24 days of DOC enrichment under concomitant heat stress (32 ^{o}C) and 2) an experimental manipulation of algal communities in the coral model Aiptasia to generate distinct holobiont combinations of host and algal strains as well as aposymbiotic (i.e., algal-free) hosts. Our findings revealed that excess DOC alone had no effect on the diazotroph and denitrifier abundance in octocorals at ambient temperature. However, excess DOC and concomitant heat stress resulted in a contrasting microbiome response between diazotrophs (increased abundance) and denitrifiers (host-specific unaltered or decreased abundance) in octocorals. Such increased diazotroph abundance without equivalent increased denitrifier abundance in octocoral holobionts may disrupt the nitrogen limitation required for maintaining the symbiotic relationship. The presence of algal symbionts increased denitrifier

abundance by up to 22-fold in Aiptasia holobionts. As increase of denitrifier abundances aligned with the superior photosynthetic performance of the inoculated algae and the identification of mostly heterotrophic denitrifiers in photosymbiotic Aiptasia, the photosynthetic carbon released by Symbiodiniaceae may be an important energy source for denitrifier communities. Thereby, I propose a positive feedback loop of photosynthetic Carbon, Denitrifier, Nitrogen limitation, and Symbiosis (PDNS) in the photosymbiotic holobiont, which may effectively contribute to maintaining the cnidarian-algal symbiosis in the unperturbed holobiont.

This thesis extends our understanding on the ecological significance of nitrogen cycling microbes in cnidarian holobionts and emphasizes the importance of algae-prokaryote interactions during this process. For a better understanding on the function of nitrogen cyclers underlying the cnidarian-algal symbiosis, future research should combine microbiome manipulations with functional gene knockouts in selected denitrifier isolates. At an ecological scale, comparing dynamics of nitrogen cycling prokaryotes in a range of marine cnidarian hosts, including octocorals, under environmental change may give new insights into the ecological drivers of novel reef ecosystems in which reef-building corals are largely replaced as dominant ecosystem engineers.

Zusammenfassung

Korallen bilden eine symbiotischen Beziehungen mit eukaryotischen und prokaryotischen Mikroben, die zusammen als "Korallenholobiont" bezeichnet werden. Ein effizienter Nährstoffaustausch zwischen Korallenwirt und photosynthetischen Symbiodiniaceae begünstigent die hohe Produktivität der Korallen in oligotrophen Gewässern. Dieses erfordert jedoch eine Stickstofflimititaion innerhalb des Holobionten. Eine Schlüsselrolle spielen hierbei Stickstoff zirkulierende Prokayoten (Baktereien und Archaeen). Vorallem in Octocorallia ist wenig über die azzoziierten Stickstoffzirkulierenden Prokaryoten bekannt, Vermutet wird, dass Beispeilsweise Denitrifikanten (d. h. Prokaryoten, die Nitrat/Nitrit zu N_2 reduzieren können) einen wichtigen Beitrag leisten eine Stickstofflimitation im Holobiont aufrechtzuerhalten. Diese Arbeit soll das derzeitige Wissen über stickstoffverarbeitende Prokaryoten in Korallenholobionten erweitern. Es wurden hierfür drei Forschungsziele verfolgt: 1) Untersuchung diazothropher Prokaryoten (d.h., Prokaryoten, die in der Lage sind, N_2 in Ammoniak umzuwandeln) und Denitrifikaten in zwei Octacoallinen Arten: die mixotrophe Weichkoralle *Xenia umbellata* und die autotrophe Gorgonie *Pinnigorgia flava* in Stressor Experimenten; 2) Untersuchung der der Wirts- und Symbiondinium Spezififischen Gemeinschaft an Dentritifikaten; 3) Charakterisierung der Gemeinschaft an Dentritifikaten imHolobionten und Identifizierung ihrer treibenden Faktoren. Die experimentelle Arbeit bestand aus einem multidisziplinären Ansatz, der physiologische, molekulare und bioinformatische Methoden kombiniert und im Laufe von zwei Experimenten in Aquarien durchgeführt wurde: 1) ein 45-Tage-Experiment, bestehend aus 21 Tagen DOC-Anreicherung bei 26 °C, gefolgt von 24 Tagen DOC-Anreicherung unter gleichzeitigem Hitzestress (32 °C), und 2) eine experimentelle Manipulation der Algengemeinschaften im Korallenmodell Aiptasia, um verschiedene Holobionten-Kombinationen aus Wirts- und Algenstämmen sowie aposymbiotische (d. h. algenfreie) Wirte zu erzeugen. Unsere Ergebnisse zeigten, dass ein Überschuss an DOC allein, ohne HItzestress, keine Auswirkungen auf die Häufigkeit von Diazotrophen und Denitrifikanten in Octocorallia hat. Überschüssigesr DOC mit gleichzeitigem Hitzestress führten jedoch zu einer gegensätzlichen Reaktion des Mikrobioms von Diazotrophen (erhöhte Abundanz) und Denitrifikanten (wirtsspezifisch unveränderte oder verringerte Abundanz). Eine solche Zunahme der Diazotrophen ohne eine entsprechende Zunahme der Denitrifikanten in den Holobionten der Oktokorallen könnte die für die Aufrechterhaltung der symbiotischen Beziehung erforderliche Stickstofflimitation stören. Das Vorhandensein von Algensymbionten erhöhte die Denitrifikanten Abundanz in

Aiptasia-Holobionten um das bis zu 22-fache. Da die Zunahme der Denitrifikanten Abundanz mit der photosynthetischen Leistung der beimpften Algen im Aiptasia übereinstimmt, könnte der von Symbiodiniaceae freigesetzte photosynthetische Kohlenstoff eine wichtige Energiequelle für Denitrifikantengemeinschaften sein. Im Rahmen meiner ERgebnisse schlage ich eine positive Rückkopplungsschleife von photosynthetischem Kohlenstoff, Denitrifikanten, Stickstofflimitierung und Symbiose (PDNS) im photosymbiotischen Holobionten vor, die wirksam dieAufrechterhaltung der Nesseltier-Algen-Symbiose im ungestörten Holobionten beitragen kann.

Diese Arbeit erweitert unser Wissen über die ökologische Bedeutung von stickstoffverarbeitenden Mikroben in Nesseltier-Holobionten und unterstreicht die Bedeutung von Algen-Prokaryoten-Interaktionen während dieses Prozesses. Für ein besseres Verständnis der Funktion der Stickstoff-Cycler, die der Nesseltier-Algen-Symbiose zugrunde liegen, sollten zukünftige Forschungen Mikrobiom-Manipulationen mit funktionalen Gen-Knockouts in ausgewählten Denitrifikanten-Isolaten kombinieren. Auf ökologischer Ebene könnte der Vergleich der Dynamik von Stickstoff zirkulierenden Prokaryoten in einer Reihe von marinen Nesseltieren, einschließlich Oktokorallen, unter veränderten Umweltbedingungen neue Einblicke der ökologischen Parameter neuartiger Riffökosysteme geben, in denen riffbildende Korallen als dominante Ökosystemingenieure weitgehend ersetzt werden.

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List of Abbreviations

Abbreviation	Full name
‰	Parts per mille, the units in which δ values are expressed
$\delta^{15}N$	Variation of the ^{15}N:^{14}N ratio of a sample relative to the ratio of the standard
$\delta^{13}C$	Variation of the ^{12}C:^{12}C ratio of a sample relative to the ratio of the standard
ΔCt	Delta Cycle threshold
ΔΔCt	Delta Delta Cycle threshold
°C	Degrees Celsius
16S rRNA	16S ribosomal RNA
ANOSIM	Analysis of similarities
ANCOM-BC	Analysis of Compositions of Microbiomes with Bias Correction
ANOVA	Analysis of variance
ASV	Amplicon sequence variants
Corg	Total organic carbon
Ct	Cycle Threshold
C	Carbon
C:N ratio	Carbon to nitrogen ratio
Diazotroph	Prokaryotes that fix molecular dinitrogen gas into ammonia
Denitrifier	Prokaryotes that reduce nitrate/nitrite to molecular dinitrogen gas
DNA	Desoxyribonucleic acid
DIN	Dissolved inorganic nitrogen
DOC	Dissolved organic carbon
DEPC	Diethyl pyrocarbonate
e.g.	An abbreviation of exempli gratia (“for example”)
Fv/Fm	Maximum (fluorescence) quantum yield
HSD	Honestly significant difference
i.e.	An abbreviation of id est (“that is”)
NMDS	Non-metric multidimensional scaling
N	Nitrogen
N_2	Dinitrogen
NH_4^+	Ammonium
NO_3^-	Nitrate
NO_2^-	Nitrite
N:P ratio	Nitrogen to phosphorus ratio
NCBI	National Center for Biotechnology Information
nifH	Nitrogenase
nirS	Nitrite reductase
ORF	Open reading frames
O_2	Oxygen
PERMANOVA	Permutational multivariate analysis of variance
PAM	Pulse-amplitude modulation
PCR	Polymerase chain reaction
PO_4^{3-}	Phosphate
PCA	Principal component analysis
P	Phosphorus
qPCR	Quantitative PCR (real-time PCR)
R	Respiration
RNA	Ribonucleic acid

SRA	Sequence Read Archive
SST	Sea surface temperature
sp.	Species
spp.	Species pluralis
SD	Standard deviation
SE	Standard error
TOC	Total organic carbon
T	Temperature
TC_{org}	Total organic carbon content
TN	Total nitrogen content

List of Figures

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 in % of the total work load (up to 100% for each of the following categories):

Chapter 2: Excess labile carbon promotes diazotroph abundance in heat-stressed octocorals

Experimental concept and design:	75%
Experimental work and/or acquisition of (experimental) data:	90%
Data analysis and interpretation:	90%
Preparation of Figures and Tables:	100%
Drafting of the manuscript:	100%

Chapter 3: Contrasting microbiome dynamics of putative denitrifying bacteria in two octocoral species exposed to dissolved organic carbon (DOC) and warming

Experimental concept and design:	75%
Experimental work and/or acquisition of (experimental) data:	90%
Data analysis and interpretation:	90%
Preparation of Figures and Tables:	90%
Drafting of the manuscript:	100%

Chapter 4: Presence of algal symbionts affects denitrifying bacterial communities in the coral model Aiptasia

Experimental concept and design:	60%
Experimental work and/or acquisition of (experimental) data:	90%
Data analysis and interpretation:	90%
Preparation of Figures and Tables:	100%
Drafting of the manuscript:	100%

Date: 01 July 2022 Signatures:

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Chapter 1 Introduction

1 General Introduction

1.1 State of the art

1.1.1 Nutrient exchange in the coral-algal symbiosis

Coral reefs are hotspots of biodiversity and productivity on Earth (1, 2). Yet, the productive coral reefs often flourish in nutrient-poor marine environments (1, 3). This contradiction is coined the “Darwin Paradox” in reference to its first observer (1, 4). The efficient nutrient recycling of benthic reef organisms may help explain this paradox (3, 4). In particular, the mutualistic nutrient exchange between the coral host and its endosymbiotic dinoflagellate algae of the family Symbiodiniaceae is the key to the ecological success of corals and the reefs they support (1, 3, 5). Indeed, the Symbiodiniaceae that live within gastrodermal cells of the coral host, can meet up to 95% of the carbon requirement of their animal partner, mostly in the form of carbohydrates and low–molecular-weight lipids (6-8). In turn, the coral host provides inorganic nutrients and CO_2 to the Symbiodiniaceae to facilitate algal growth and photosynthesis (3, 5, 9).

The maintenance of the coral-algal symbiosis is heavily reliant on the continuous and limited availability of nitrogen for Symbiodiniaceae (10, 11). Excess nitrogen availability could rapidly promote algal growth, resulting in a self-retention of photosynthetic products (10, 12). As a consequence, the entire symbiotic relationship between corals and Symbiodiniaceae may collapse and result in host animal starvation (10, 12, 13). Given the spatial and seasonal nutrient fluctuations in coral reef waters, the stability of coral-algal symbiosis may therefore depend on its ability to regulate the nitrogen availability for Symbiodiniaceae (4, 10). Aside from Symbiodiniaceae, a range of prokaryotic microbiota are associated with corals; this multipartite symbiotic organism is collectively referred to as the "coral holobiont" (14, 15). Nitrogen cycling microbes, among others, have garnered considerable attention due to their potential importance for regulating the nitrogen availability within the coral holobiont (4, 16-22).

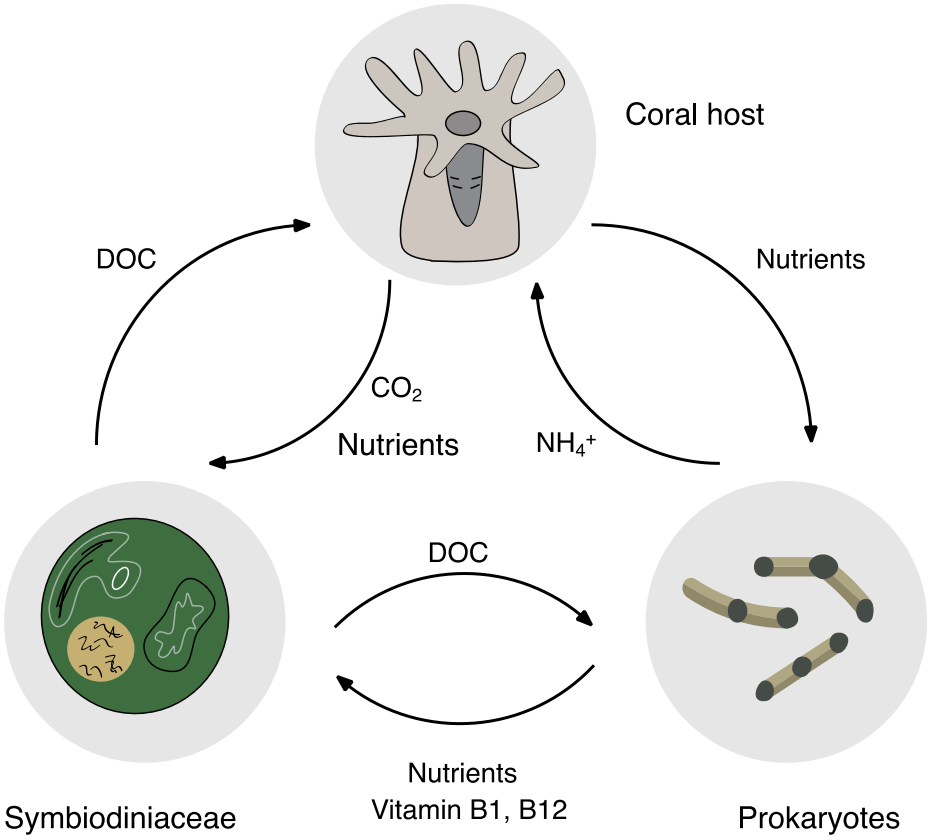

Fig. 1.1 Schematic illustration of the major nutrient exchange pathways in the coral holobiont facilitated by the tripartite interactions between coral host, Symbiodiniaceae, and the associated prokaryotes. These interactions and metabolic pathways occur across all three holobiont members. Photosynthetic fixed carbon from Symbiodiniaceae is passed to both the coral host and the prokaryotic microbiome. In turn, inorganic nutrients derived from coral host metabolism can support Symbiodiniaceae growth and potentially be utilized by nitrogen cycling prokaryotes. Prokaryote-derived inorganic nitrogen sources may contribute to satisfying the nutrient requirements of both coral host and Symbiodiniaceae. Abbreviation: CO_2 = Carbon dioxide; DOC = Dissolved organic carbon; NH_4^+ = Ammonia. Adapted from Bourne et al., 2016 and Iha et al., 2021 (77, 78).

1.1.2 Nitrogen cycling microbes and their role in stable coral holobionts

Nitrogen cycling microbes in the coral holobiont are mostly made up of a broad range of prokaryotes capable of fixing N_2 into ammonia (i.e., diazotrophs), oxidizing ammonium to nitrite/nitrate (i.e., nitrifiers), and reducing nitrate/nitrite to N_2 (i.e., denitrifiers) (4, 17, 18, 20, 22, 23). Cyanobacteria were the first discovered diazotrophs in corals, implying that they may be an important additional nitrogen source in the holobiont (16). Recent studies employing in-depth sequencing of the nitrogenase *nifH* gene have revealed that Cyanobacteria, Alpha- and Gamma-proteobacteria dominated the diazotroph community of reef-building coral holobionts (24, 25). Diazotroph abundances in reef-building corals are host-specific and broadly align with the trophic strategy of host animals (26). It has hence been proposed that the high abundance of diazotrophs in highly autotrophic corals may compensate for the low nitrogen availability due to limited heterotrophy (9, 26). Specifically, diazotroph-derived nitrogen (DDN) sources have been suggested to be particularly important for the nitrogen budget of coral holobionts in nutrient-poor environments (27). As such, diazotrophs may support the productivity of coral holobionts in oligotrophic tropical waters (19, 28).

Coral reefs are subject to seasonal dynamics in nutrient fluctuations (21, 28, 29). Symbiodiniaceae are able to take up ammonia, nitrate, and nitrite from the surrounding seawater, thus excess environmental nitrogen availability may potentially threaten the stability of coral-algal symbiosis (4, 30). In this context, denitrifiers, i.e., prokaryotes capable of reducing nitrate/nitrite to N_2, may play a crucial role in alleviating excessive nitrogen stressor in the coral holobiont (4, 31). Denitrification activity has been reported for the first time on coral reefs about 30 years ago (32). Recently, the presence of denitrifiers within the coral holobiont has also been confirmed (33). This work, which used nitrite reductase *nirS* gene clone library construction and sequencing, demonstrated that coral-associated denitrifiers are comprised by a group of prokaryotes belonging to the Alpha-, Beta-, and Gammaproteobacteria (33). According to a recent study, the abundance and activity of denitrifiers in reef-building coral holobionts were positively correlated with each other, and host-specific (31). Denitrifier abundance (thereby denitrification activity) further aligned with Symbiodiniaceae density and nitrate availability in seawater, and may reflect their ability to alleviate nitrogen accumulation within the coral holobiont (4, 21). Thereby, nitrogen cycling microbes may contribute to

maintaining the nitrogen limitation, required for the functioning of coral holobionts under undisturbed environmental conditions.

1.1.3 Coral holobionts under environmental change

Global and local anthropogenic change are threatening the stability of coral holobionts and reefs they support (34, 35). Ocean warming, as a consequence of global climate change, is the primary cause of bleaching in reef-building corals, i.e., the whitening of coral due to the loss of Symbiodiniaceae or the degradation of photosynthetic pigment (34, 35). Over the last 50 years, seawater surface temperatures (SST) have increased at a pace of 0.11 degrees per decade, and are predicted to rise by 1.5^{o}C between the years 2030 and 2050 (36). Although different species of reef-building corals exhibit different thermal bleaching thresholds (37), 50-60% of global coral reefs are unlikely to survive a global warming of 1.5^{o}C (38). Apart from global climate change (34, 35), local stressors such as overfishing, pollution, and poor coastal water quality are also considered major factors for the declining live coral cover on reefs (39, 40). Prior studies have demonstrated that eutrophication can rapidly cause bleaching in reef-building corals and promotes reef degradation (41-47). Organic eutrophication, e.g., excess dissolved organic carbon (DOC) loading, has been shown to disrupt the symbiotic relationship between the coral host and microbial communities, resulting in increased coral mortality (45, 46).

The consequences of global climate change (e.g., ocean warming) and local activities (e.g., organic eutrophication) on reef-building coral holobionts include not just the loss of algal symbionts, but also remarkable changes in nitrogen cycling microbial communities (47-50). Ocean warming was shown to cause significant shifts in diazotroph community structure of reef-building coral holobionts (49, 50), and to stimulate diazotroph abundance and activity (48). Likewise, excess DOC promoted the proliferation of diazotrophs in reef-building coral *Pocillopora verrucosa*, resulting in increased nitrogen availability of Symbiodiniaceae (47). In summary, excess nitrogen availability can threaten the functioning of reef-building coral holobionts in two ways. First, excess nitrogen could rapidly promote the growth of algal symbionts, resulting in a reduced translocation of photosynthetic carbon to their host corals (10, 51). Second, raised nitrogen levels may cause algal symbionts to transition from a nitrogen-

limited to a phosphorus-starved state (44). In such a state, the phospholipids in the thylakoid membranes of algal chloroplast are replaced by sulfolipids, which weakens the stability of the thylakoid membrane, rendering the photosynthetic system more vulnerable and lowering the bleaching threshold of corals (52, 53). Taken together, nitrogen cycling microbes are considered integral players in overall coral holobiont functioning. However, their beneficial role in holobiont functioning may be undermined by global and local environmental changes (47-50, 54, 55).

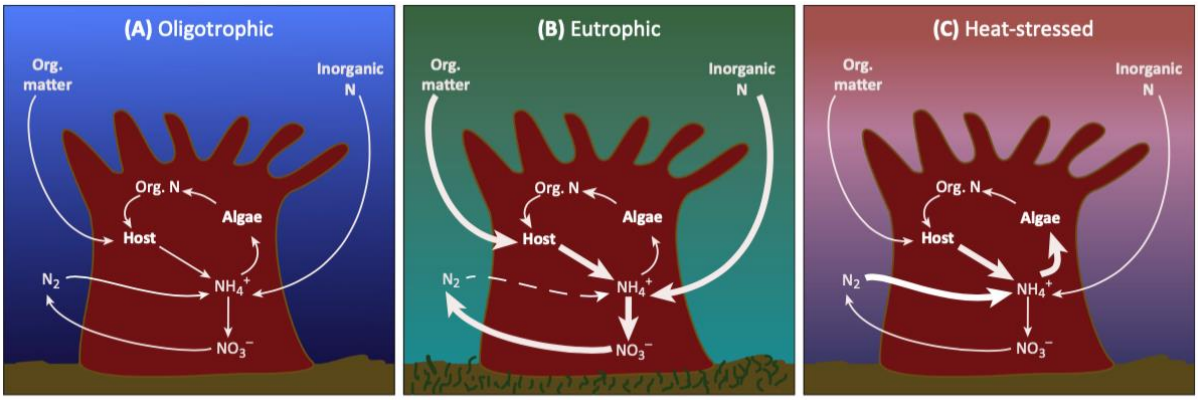

Fig 1.2 Hypothesized microbial-mediated nitrogen cycling in the coral holobiont. Representation of major nitrogen cycling pathways in the coral holobiont under (A) oligotrophic, (B) eutrophic, and (C) heat stress. Bold arrows indicate potential relative amplification of pathways compared to an oligotrophic scenario. Broken arrows indicate potential relative diminution of pathways compared to an oligotrophic scenario. Microbial-mediated nitrogen cycling may help to regulate algal growth under oligotrophic and eutrophic conditions, but may lead to elevated nitrogen availability under heat-stressed conditions. A representation of the localization of individual processes within the holobiont is omitted because sufficient knowledge is lacking to date. Abbreviation: N = nitrogen; N_2 = atmospheric dinitrogen; Org. = organic. Figure source: Rädecker et al., 2015 (4).

1.1.4 Octocorals as winners of reef phase shifts

The degradation of coral reefs under environmental change is not reflected in the disappearance of all benthic organisms, but rather in the major restructuring of coral reef communities (56). Octocorals (most of them are not reef-building), including soft corals and gorgonians, are the second most common benthic reef organisms on many Indo-Pacific and Caribbean reefs after the reef-building corals (57, 58). Unlike reef-building corals, most octocoral species have shown remarkable resistance to different global and local environmental disturbances, which may explain the observed global phase shifts from reef-building coral to octocoral dominance (**Fig.1.3**) (59-72). Importantly, octocorals also have evolved intricate and complex interactions with a diverse range of prokaryotic microbiota, some of which are involved in nutrient cycling (73). For instance, diazotroph activity has been detected in the soft corals *Sarcophyton* sp. and *Xenia* sp. from the Northern Red Sea (28), and the presence of denitrifiers has been confirmed in the soft corals *Xenia* sp. and *Alcyonium* sp. (33, 74). As such, nitrogen cycling microbes may be common members of the octocoral microbiome (73). However, the potential role of nitrogen cycling microbes in octocoral holobiont functioning and resistance to a changing environment is largely unknown.

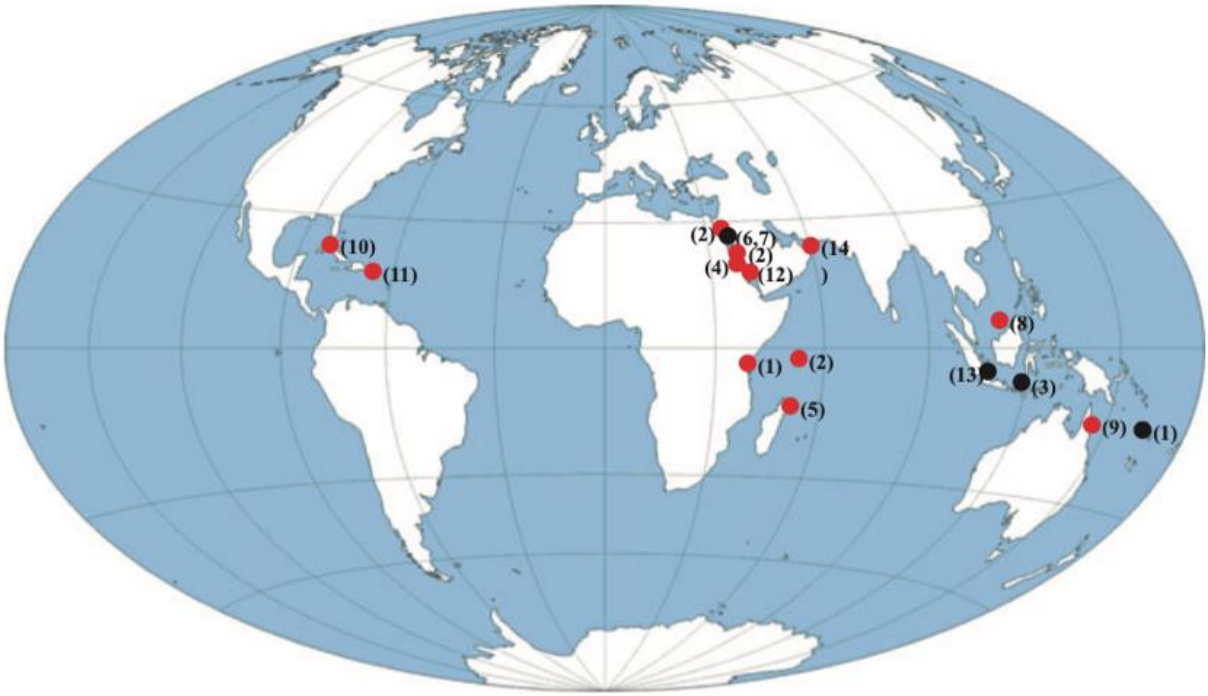

Fig. 1.3 Exemplary overview of reported phase shifts from reef-building coral to octocoral dominance caused by natural disturbances and anthropogenic activities. The natural disturbances include bleaching, storms, cyclones, El Ni o, or crown-of-thorns starfish (red dots). Anthropogenic activities include blast fishing, pollution, and tourism (black dots). Numbers adjacent to the marks indicate the literature source: (1) Hoffmann (2002)(79), (2) Wilkinson (2002)(80), (3) Fox et al. (2003)(81), (4) Reinicke et al. (2003)(82), (5) Stobart et al. (2005)(83), (6) Al-Zibdah et al. (2007)(84), (7) Tilot et al. (2008)(85), (8) Wood and Dipper (2008)(86), (9) Wakeford et al. (2008)(87), (10) Ruzicka et al. (2013)(88), (11) Lenz et al. (2015)(89), (12) Bruckner and Dempsey (2015)(90), (13) Baum et al. (2015)(91), and (14) Coles et al. (2015)(92). Figure source: Schubert et al., 2016 (93).

1.2 Thesis premise

1.2.1 Knowledge gaps and research hypothesis

This thesis aims to address the following four knowledge gaps and related research hypotheses as outlined below:

Research gap 1- Constant and limited nitrogen availability is the foundation for stable mutualistic nutrient exchange of coral-algal symbiosis (10, 12, 13). Recent studies have shown that both global warming and local dissolved organic carbon (DOC) loading could promote the growth and activity of diazotrophs in reef-building corals, resulting in excess nitrogen availability that threatens the functioning of the holobiont (47, 48). However, it remains unclear whether diazotrophs are associated with taxonomically and trophically distinct octocorals, and how they (if present) respond to excess DOC and its interaction with heat stress.

Research hypothesis 1: Similar to reef-building corals, excess DOC as readily accessible energy promotes diazotroph abundance in octocorals. Concomitant heat stress facilitates the stimulating effect of excess DOC on diazotroph proliferation.

Research gap 2- Denitrifiers may play a crucial role in coral holobiont functioning owing to their potential to maintain nitrogen limitation of the holobiont. Octocorals have shown some of the highest denitrification activities among benthic reef organisms (74). However, it is unknown how the denitrifier community in octocorals responds to global (e.g., ocean warming) and local environmental disturbances (e.g., excess DOC), and which microbes are potentially involved in this process.

Research hypothesis 2: Excess DOC and its interaction with heat stress promote denitrifier abundance to counterbalance the elevated diazotroph-derived nitrogen availability in the octocoral holobiont. The increasing denitrifier abundance is driven by a few denitrifier taxa that are distinct in different octocoral holobionts.

Research gap 3- As most denitrifiers are heterotrophic (75), the photosynthetically-derived organic carbon of Symbiodiniaceae may be an important energy source for them. To date, the potential interactions between coral host, Symbiodiniaceae, and denitrifiers remain largely speculative. The respective contribution of host and algal identity to the structuring of the denitrifier community in the cnidarian holobiont is still unknown.

Research hypothesis 3: The presence of algal symbionts promotes denitrifier abundance in the cnidarian holobiont.

Research gap 4- Nitrogen cycling microbes are considered key players in coral (by extension, cnidarian) holobiont functioning. It is hence imperative to identify members of the different functional groups of nitrogen cyclers. Previously, coral-associated diazotrophs have been characterized by in-depth sequencing of the nitrogenase *nifH* gene (24, 25, 49, 76). In contrast, next-generation sequencing (NGS) of the functional genes associated with denitrification, such as the nitrite reductase *nirS* gene has not yet been applied to characterize the denitrifier community in cnidarian holobionts. Consequently, the abiotic and holobiont-associated biotic drivers of denitrifier communities in the cnidarian holobiont remain largely unknown.

Research hypothesis 4: Denitrifier community in the cnidarian holobiont is highly dominated by a few heterotrophic prokaryotes, thus photosynthetic carbon availability is one of the main driving forces for denitrifier patterns.

1.2.2 Contribution of the thesis

To address the above knowledge gaps, I conducted two experiments that yielded three independent research chapters of this thesis. First, I assessed the dynamics of diazotroph abundances in two common but trophically distinct octocoral species under global (achieved by heat stress) and local environmental change (achieved by DOC dosing) (**Hypothesis 1, Chapter 2**), and then moved to denitrifier dynamics with main focus on identifying the putative key players and (a)biotic drivers (**Hypothesis 2, Chapter 3**). Finally, I tested the effect of algal and host identity on the structuring of denitrifier communities in the coral holobiont (using the

laboratory coral model organism "Aiptasia") (**Hypothesis 3 and 4, Chapter 4**). The specific contributions of each thesis chapter are detailed below.

In Chapter 2, I investigated the relative diazotroph abundances in two octocoral species with contrasting trophic strategies: the soft coral *Xenia umbellata* (Lamarck, 1816) and the gorgonian *Pinnigorgia flava* (Nutting, 1910). I first evaluated eight different *nifH* primer pairs that have been previously used to detect diazotroph communities in reef-building corals, and then used the successful primer pair to quantify the relative diazotroph abundance in both octocorals in a 45-day aquarium experiment that encompassed the first 21 days of excess DOC (10 mg L^{-1}, 20 mg L^{-1}, and 40 mg L^{-1}), and the last 24 days of excess DOC and concomitant warming. I addressed the following research questions: 1) Do diazotroph abundances in octocorals (if present) align with the trophic strategy of host animals? 2) Do excess DOC, as a readily available energy source, promote diazotroph abundance, and 3) How does excess DOC and concomitant warming affect relative diazotroph abundance?

Chapter 3 used the same experimental design and coral species as Chapter 2, with the exception that the research focus was shifted from diazotrophs to denitrifiers, which have received considerably less attention in symbiotic cnidarian holobionts so far. I first employed qPCR of the *nirS* gene to quantify the denitrifier communities in *X. umbellata* and *P. flava* holobionts under undisturbed conditions, specifically under excess DOC, and under excess DOC and concomitant warming. The putative denitrifier community structure and dynamics were then investigated by 16S rRNA gene sequencing and subsequent *in silico* PCR of the *nirS* gene. In addition, I measured the carbon to nitrogen (C:N) ratio and nitrogen isotope signatures in both octocoral holobionts, as well as inorganic nutrient level in surrounding seawater. Thereby, I addressed the following research questions: 1) Are denitrifiers present in the investigated octocoral holobionts? If so, are abundance and diversity of denitrifiers host-specific? 2) Does DOC, as well as its interaction with warming, influence the abundance and community structure of denitrifiers? 3) Do denitrifier community dynamics align with the nutritional status of octocoral holobionts?

In Chapter 4, I harnessed the coral model organism "Aiptasia" and used qPCR of the *nirS* gene to quantify the denitrifier abundance in a full-factorial design, combining two

Aiptasia host strains in an aposymbiotic state or symbiotic with two different Symbiodiniaceae clonal lineages. In addition, I performed NGS of *nirS* gene amplicon to achieve an in-depth characterization of denitrifier communities in the cnidarian holobiont. I also assessed the physiological and nutritional status of holobionts by measurements of symbiont density, photosynthetic efficiency, and C:N ratio. I addressed the following research questions: 1) Does the presence of algal symbionts influence the denitrifier community of Aiptasia holobionts? 2) If so, does algal or host identity matter? 3) Are patterns in denitrifier abundance and community structure reflecting the nutritional status of holobionts?

1.2.3 Thesis outline

This thesis consists of a general introduction (Chapter 1), three chapters presenting the Ph.D. research work (Chapters 2-4), and a general discussion (Chapter 5). Chapters 2-4 consist of published manuscripts and manuscripts intended for publication in international peer-reviewed journals. Four co-authored publications related to this thesis are listed below, with the abstracts of them presented in the Annex of the thesis.

1.2.4 Publication overview

Chapter 2 in full is in preparation for the journal *Royal Society Open Science*:

1. **Nan Xiang**, Achim Meyer, Claudia Pogoreutz, Nils Rädecker, Christian R. Voolstra, Christian Wild, and Astrid Gärdes. Excess labile carbon promotes diazotroph abundance in heat-stressed octocorals. In preparatio for the journal *Royal Society Open Science.*

This project was initiated by Nan Xiang (70%), Prof. Christian Wild (20%), and Prof. Astrid Gärdes (10%). The experimental design was developed by Nan Xiang (75%) and Prof. Christian Wild (25%). Sample processing was conducted by Nan Xiang (90%) with the assistance of Dr. Achim Meyer (10%). Data visualization and statistical analysis were conducted by Nan Xiang (90%) with improvement suggestions from Dr. Claudia Pogoreutz

(5%) and Dr. Nils Rädecker (5%). The manuscript was written by Nan Xiang with improvements from all contributing authors.

Chapter 3 in full has been published in the journal *Applied and Environmental Microbiology*.

2. **Nan Xiang**, Christiane Hassenrück, Claudia Pogoreutz, Nils Rädecker, Susana Marcela Simancas-Giraldo, Christian R. Voolstra, Christian Wild, and Astrid Gärdes. (2022). Contrasting microbiome dynamics of putative denitrifying bacteria in two octocoral species exposed to dissolved organic carbon (DOC) and warming. DOI: https://doi.org/10.1128/AEM.01886-21.

This project was initiated by Nan Xiang (50%), Prof. Christian Wild (25%), and Prof. Astrid Gärdes (25%). The experimental design was developed by Nan Xiang (75%) and Prof. Christian Wild (25%). Sample processing was conducted by Nan Xiang (95%) and Susana Marcela Simancas-Giraldo (5%). Sequence analysis was conducted by Nan Xiang (70%) and Dr. Christiane Hassenrück (30%). Data visualization and statistical analysis were conducted by Nan Xiang (80%) with improvement suggestions from Dr. Claudia Pogoreutz (10%), and Dr. Nils Rädecker (10%) . The manuscript was written by Nan Xiang with improvements from all contributing authors.

Chapter 4 in full is under review in the journal *ISME Communications.*

3. **Nan Xiang**, Nils Rädecker, Claudia Pogoreutz, Anny Cárdenas, Anders Meibom, Christian Wild, Astrid Gärdes, and Christian R. Voolstra. Presence of algal symbionts affects denitrifying bacterial communities in the coral model Aiptasia. Under review in the journal *ISME Communications.*

This project was initiated by Nan Xiang (30%), Dr. Nils Rädecker (30%), Dr. Claudia Pogoreutz (20%), Prof. Astrid Gärdes (10%), and Prof. Christian Voolstra (10%). The experimental design was developed by Nan Xiang (20%), Dr. Nils Rädecker (50%), and Dr.

Claudia Pogoreutz (30%). Sample processing was conducted by Nan Xiang (85%), Dr. Nils Rädecker (10%), and Dr. Claudia Pogoreutz (5%). Sequence analysis was conducted by Nan Xiang (70%) with the guidance of Dr. Anny Cárdenas (25%) and Prof. Christian R. Voolstra (5%). Data visualization and statistical analysis were conducted by Nan Xiang (90%) with the guidance of Dr. Anny Cárdenas (10%). The manuscript was written by Nan Xiang with improvements from all contributing authors.

Co-author contributions during the Ph.D.:

1. Svea Vollstedt, **Nan Xiang**, Susana Marcela Simancas-Giraldo, and Christian Wild. Organic eutrophication increases resistance of the pulsating soft coral *Xenia umbellata* to warming. Published in the journal *PeerJ*. (2020). DOI: http://dx.doi.org/10.7717/peerj.9182.

The dissertation author was involved in experimental design, project coordination, aquaria maintenance, assistance in statistical analysis and data visualization, and manuscript improvement.

2. Susana Marcela Simancas-Giraldo, **Nan Xiang**, Meghan Moger Kennedy, Rassil Nafeh, Edoardo Zelli, and Christian Wild. Photosynthesis and respiration of the soft coral *Xenia umbellata* respond to warming but not to organic carbon eutrophication. Published in the journal *PeerJ*. (2021). DOI: https://doi.org/10.7717/peerj.11663.

The dissertation author was involved in experimental design, project coordination, aquaria maintenance, and manuscript improvement.

3. Claudia Pogoreutz, Clinton A Oakley, Nils Rädecker, Anny Cárdenas, Gabriela Perna, **Nan Xiang**, Lifeng Peng, Simon K Davy, David K Ngugi, and Christian R Voolstra. Coral holobiont cues prime *Endozoicomonas* for a symbiotic lifestyle. Published in *The ISME Journal*. (2022). DOI: https://doi.org/10.1038/s41396-022-01226-7.

The dissertation author was involved in molecular experiments of PCR and qPCR.

4. Edoardo Zelli, Susana Marcela Simancas-Giraldo, **Nan Xiang**, Claudia Dessì, Nadim Daniel Katzer, and Christian Wild. Individual and combined effect of organic eutrophication and ocean warming on the ecophysiology of the gorgonian *Pinnigorgia flava*. In revision for the journal *PeerJ*. (2022).

The dissertation author was involved in experimental design, project coordination, aquaria maintenance, and manuscript improvement.

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Chapter 2 Excess labile carbon promotes diazotroph abundance in heat-stressed octocorals

Excess labile carbon promotes diazotroph abundance in heat-stressed octocorals

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Running Head: DOC boosts Diazotrophs in Heat-stressed Octocorals

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[++]These authors contributed equally to this work.

2.1 Abstract

Constant and limited nitrogen availability is the root of a stable coral-algal symbiosis. Hence, diazotrophs, i.e., prokaryotes capable of fixing N_2 into ammonia, are widely considered key players in the fitness and functioning of the coral holobiont. Recent studies in reef-building coral holobionts have shown that labile carbon enrichment or heat stress may overstimulate diazotrophs, resulting in excess nitrogen availability. However, the (a)biotic driver for diazotroph communities in octocorals is still poorly understood. Here, we investigated the dynamics of diazotroph abundance (assessed via qPCR quantification of relative *nifH* gene copy numbers) in two symbiotic octocorals with distinct trophic strategies, the mixotrophic soft coral *Xenia umbellata* and the highly autotrophic gorgonian *Pinnigorgia flava* to: a) three levels of dissolved organic carbon (DOC) enrichment for 21 days, followed by (b) combined DOC enrichment and heat stress over the course of 24 days. In the absence of heat stress, relative abundances of diazotrophs in *X. umbellata* and *P. flava* were unaffected by DOC enrichment. During subsequent heat stress, however, excessive DOC enrichment (higher than 20 mg L^{-1}) significantly increased the relative abundance of diazotrophs by 6 folds in *X. umbellata* and 4 folds in *P. flava*, respectively. Our data suggest that heat stress may stimulate the effect of DOC enrichment on diazotroph proliferations in octocorals. Thereby, improving coastal water quality by reducing DOC loading to coral reefs may effectively help octocoral holobionts to cope with the effect of global warming.

2.2 Introduction

Coral reefs typically thrive in oligotrophic environments and are among the most diverse and productive ecosystem on earth (1). The efficient nutrient recycling between holobiont members, i.e., animal host, photosynthetic dinoflagellates of the family Symbiodiniaceae, and a suite of diverse prokaryotic microbiome, is the key to support this success (2, 3). Indeed, diazotrophs, i.e., prokaryotes capable of converting atmospheric dinitrogen into ammonia, can provide a "new" nitrogen source for the holobiont, sustaining the productivity of corals when environmental nutrient availability is low (4, 5). Diazotrophs are ubiquitous members of reef-building coral and octocoral (usually not reef-building) holobionts with pronounced host-dependent differences in their abundance, activity, and community structure (5-9).

Global and local environmental change threaten to destabilize the functioning of coral holobionts and the reefs they support (10-13). Importantly, diazotrophs in reef-building corals are susceptible to changing environmental conditions (3, 8, 9, 13-18). Previous studies document that coral bleaching, the stress-induced breakdown of a stable holobiont functioning, not only results in the loss of Symbiodiniaceae but also coincide with the opportunistic proliferation of diazotrophs (13, 19, 20). The increase in diazotroph-derived nitrogen (DDN) stimulated by heat stress or DOC enrichment has been suggested to partly lead to the destabilization of nitrogen limitation, required for the efficient nutrient exchange between coral host and Symbiodiniaceae (13, 14, 19, 20).

Similar to reef-building corals, symbiotic octocorals also rely on the intimate metabolic interactions between holobiont members (21, 22). However, octocorals are remarkably resistant to multiple environmental disturbances, such as ocean warming and acidification (23-27). As a consequence, octocorals are becoming more and more abundant on degraded coral reefs affected by anthropogenic disturbance (25, 27). Understanding the underpinning of the ecological success of octocorals could help to decipher the processes shaping emerging novel ecosystems (25, 28).

It is generally recognized that DOC enrichment or heat stress can promote the proliferation of diazotrophs in reef-building corals (13-15, 18), however the impact of them on diazotrophs in the octocoral holobiont remains unknown. Here, we thus set out an aquarium experiment over the course of 45 days, to investigate the response of octocoral-associated

diazotrophs to DOC enrichment, and its interaction with heat stress (Fig. 1). Using quantitative PCR (qPCR) of the *nifH* gene, which encodes for the iron protein of nitrogenase that catalyzes N_2 fixation, as a proxy for N_2 fixation activity (8), we compared the dynamics of relative diazotroph abundances in two symbiotic octocorals with distinct trophic strategies: the mixotrophic soft coral *Xenia umbellata* (Lamarck, 1816) and the highly autotrophic gorgonian *Pinnigorgia flava* (Nutting, 1910). We addressed the following questions: a) Do diazotroph abundances differ between octocoral species of different trophic strategies under an undisturbed condition? b) Does DOC enrichment, as a readily available energy source, promote the proliferation of diazotrophs in octocorals? c) If so, does the stimulating effect of DOC enrichment become more pronounced in heat-stressed octocorals?

2.3 Materials and Methods

2.3.1 Coral husbandry, experimental design, and sampling

Mother colonies of the soft coral *X. umbellata* and the gorgonian *P. flava* were cultivated at the indoor aquaria facility (temperature: 26 ± 0.5 °C; pH: 7.8 ± 0.2; salinity: 35 ± 3 ‰) of Marine Ecology Department at the University of Bremen for more than 2 years. This study was a companion experiment to (29) using the same coral fragments and experimental design. *X. umbellata* colonies were cut into small fragments (1-2 cm side length) and carefully fixed onto calcium carbonate holders (1×1 cm) using rubber bands. Likewise, *P. flava* colonies were fragmented into individual branches (3-4 cm length) and attached to coral holders using aquarium moss coral fix glue. A total of 120 *X. umbellata* and *P. flava* fragments each were distributed over 12 aquaria tanks (water volume 50 L each), with 10 fragments of *X. umbellata* and 10 fragments of *P. flava* in each tank. Each tank was equipped with a thermostat (3613 aquarium heater, 75 W 220-240 V, EHEIM, Germany), a pump (CompactOn 300 pump, EHEIM, Germany), a protein skimmer (SkimMarine 100; EHEIM, Germany), and LED lights (Royal Blue-matrix module and Ultra Blue White 1:1-matrix module, WALTRAt day time® LED light, Germany) at a 12:12 h light : dark cycle at an intensity of 120.8 ± 10.2 µmol quanta m^{-2} s^{-1}. Each tank was filled with freshly prepared artificial seawater (salinity: 35.40 ± 0.40 ppt; pH: 8.20 ± 0.01; temperature: 26 ± 0.4 °C) with a daily water renewal of 10% to maintain stable water parameters. To avoid excessive biofilm formation, aquaria surfaces were cleaned daily. No additional feeding was provided over the course of the experiment.

In the first phase of the experiment (**Fig. 2.1A;** total duration of 21 days), a daily dosing of glucose based on a stock solution (d-Glucose, 40 mg mL^{-1}) was performed to simulate different levels of dissolved organic carbon (DOC) loading: 3 mg L^{-1} (control); 10 mg L^{-1} (low); 20 mg L^{-1} (moderate), 40 mg L^{-1} (high). Daily measurements of total organic carbon using a TOC-L analyzer (Shimadzu, Japan) were performed as a proxy for DOC levels, and daily dosings of glucose were accordingly adjusted to achieve the desired levels (29). In the second phase (**Fig. 2.1A;** total duration of 24 days), aquaria tanks were gradually ramped up to 32°C (close to the thermal threshold of *X. umbellata*) with a 2 °C step increase every 8 days (30). DOC dosing was continued as described above during the second phase. Coral samples were collected at day 0 (baseline, before DOC enrichment), at the end of the first phase (day 21, DOC enrichment), and at the end of the second phase (day 45, combined DOC enrichment and heat stress). At each sampling time point, coral fragments (3 replicates for each treatment) were immediately flash-frozen in liquid nitrogen and subsequently stored at -80 °C freezer until further processing.

2.1.2 DNA extraction and nifH *PCR*

Frozen coral samples were ground into powder over liquid nitrogen using a sterilized mortar and pestle. The powdered samples were directly used for genomic DNA extraction with the Quick-DNA Universal Kit for Solid Tissue (ZYMO RESEARCH, USA) according to the manufacturer's instructions. The yield and quality of extracted DNA were assessed by spectrophotometry at 260 nm and 280 nm using an Infinite 200 PRO (Tecan, Austria) and visually inspected on a 1% (wt/vol) agarose gel electrophoresis (Biometra Horizon 58, Germany).

The *nifH* gene that encodes for the iron protein subunit of nitrogenase was used as a marker to assess diazotroph communities (31, 32). Eight different primer combinations (Supplementary Information, **Fig. S1**) were evaluated for their ability to amplify fragments of the *nifH* gene in the positive control (see Supplementary Method for positive control design) and DNA extracted from octocoral holobionts. Based on this, the primer pair nifH-IGK3 (5'-GCIWTHTAYGGIAARGGIGGIATHGGIAA-3') and nifH-DVV (5'-ATIGCRAAICCICCRCAIACIACRTC-3') (31) was selected for the purpose of this study. The PCR reactions consisted of: 10 µL of Taq DNA Polymerase Master Mix (VWR, USA), 1 µL GC-enhancers (Applied Biosystems, USA), 2.4 µL of 10 µM forward and 10 µM reverse primer each, 2.2 µL of nuclease-free water and 2 µL DNA template (10 ng μl^{-1}) for a total reaction

volume of 20 µL. The thermal cycling condition consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The specificity of *nifH* amplicons was validated with Sanger sequencing (StarSEQ Mainz, Germany).

2.1.3 nifH *Quantitative PCR (qPCR)*

Quantification of *nifH* gene via qPCR was used to assess the relative diazotroph abundance in the octocoral holobiont (8). The use of degenerate primers (as used here) may overestimate *nifH* abundance in the sample due to the amplification of *nifH* homologs and the unspecific amplification (32). However, in the context of the present study where the same organisms were compared under identical conditions, this amplification bias is unlikely to drive the treatment-specific effects. The qPCR condition for *nifH* gene kept the same reagent receipt and volume with the PCR outlined above. The Taq DNA Polymerase Master Mix was replaced by 2X SensiFAST master mix (bioline, Germany). The *nifH* gene copy numbers (as a proxy of diazotroph abundance) were referenced against the 16S rRNA gene copy numbers (as a reference for total bacterial abundance) amplified by the primer pair Bact-16S_784F (5'-AGGATTAGATACCCTGGTA-3') and Bact-16S_1061R (5'-CRRCACGAGCTGACGAC-3') (33), according to the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) (34). The qPCR condition for 16S rRNA gene consists of 1.0 µL forward (10 µM) and reverse primer (10 µM) each, 6.0 µL nuclease-free water, 10.0 µL 2X SensiFAST master mix, and 2.0 µL DNA template (10.0 ng μL^{-1}).

The qPCRs were performed on the CFX96 real-time detection system (Bio-Rad, USA), running with an initial polymerase activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 57 °C for 30 s (*nifH* gene) or at 60 °C for 20 s (16S rRNA gene), and extension at 72 °C for 30 s. A final extension was carried out at 72 °C for 10 s followed by a melting curve cycle from 65 °C to 95 °C with an increase of 0.5 °C every 5 s. The qPCR efficiency was validated by calibration curve generated using a serial dilution of DNA of *E. coli* (reference strain ATCC 25922) targeting the 16S rRNA gene, and DNA of the synthesized partial *nifH* sequence from *Azotobacter vinelandii* DJ targeting *nifH* gene, respectively. The qPCR efficiency was 90.33% for the *nifH* gene, and 92.31% for the 16S rRNA gene.

2.1.4 Statistical analysis

All statistical analyses were conducted in R (version 4.1.1). Plots were generated using package 'ggplot2' (35). The qPCR data were log transformed to meet the data normality checked by "Shapiro–Wilk test using package 'nortest' (36). The difference in the relative diazotroph abundance between octocoral species at each timepoint was analyzed by two-way analysis of variance (ANOVA) defining coral species and DOC enrichment as two factors. After subsetting qPCR data for *X. umbellata* and *P. flava* respectively, the data for each coral species was analyzed by two-way ANOVA defining time and DOC enrichment as two factors, followed by Tukey's honestly significant difference (HSD) as a post hoc comparison. All data were presented as mean ± SE.

2.4 Results

2.4.1 DOC enrichment does not affect relative diazotroph abundance in octocorals at ambient conditions

Using relative *nifH* gene copy numbers as a proxy for diazotroph abundances, we found that the relative abundance of diazotrophs was significantly higher in *P. flava* than in *X. umbellata* at the beginning (**Fig. 2.1B**; **Fig. 2.2**; two-way ANOVA, $F_{1,16} = 30.07$, $P < 0.001$). 21 days of DOC enrichment did not change the relative abundance of diazotrophs in both of *X. umbellata* (**Fig. 2.2**; two-way ANOVA, $F_{3,16} = 1.44$, $P = 0.268$) and *P. flava* (two-way ANOVA, $F_{3,16} = 1.72$, $P = 0.202$).

2.4.2 DOC enrichment boosts diazotroph abundance in octocorals during heat stress

Following the gradual increase in seawater temperature to 32°C (i.e., at day 45), the relative abundance of diazotrophs in *X. umbellata* (**Fig. 2.2**; two-way ANOVA, $F_{1,28} = 106.51$, $P < 0.001$) and *P. flava* (two-way ANOVA, $F_{1,28} = 12.30$, $P = 0.003$) showed a significant increase compared to day 21 (prior to heat stress). In *X. umbellata*, moderate (20 mg L^{-1}) and high DOC (40 mg L^{-1}) treatments promoted the relative abundance of diazotrophs by nearly 6-fold increase compared to the control (3 mg L^{-1}) and low DOC treatment (10 mg L^{-1}) (**Fig. 2.2**; two-way ANOVA, $F_{3,16} = 9.37$, $P = 0.001$). Similarly, a positive effect of DOC enrichment on diazotroph abundance was evident in *P. flava* albeit not significantly (**Fig. 2.2**; two-way ANOVA, $F_{3,16} = 0.79$, $P = 0.520$; relative change folds, control: 3.40 ± 1.14; low: 5.14 ± 2.10; moderate: 9.56 ± 2.53; high: 15.37 ± 3.37).

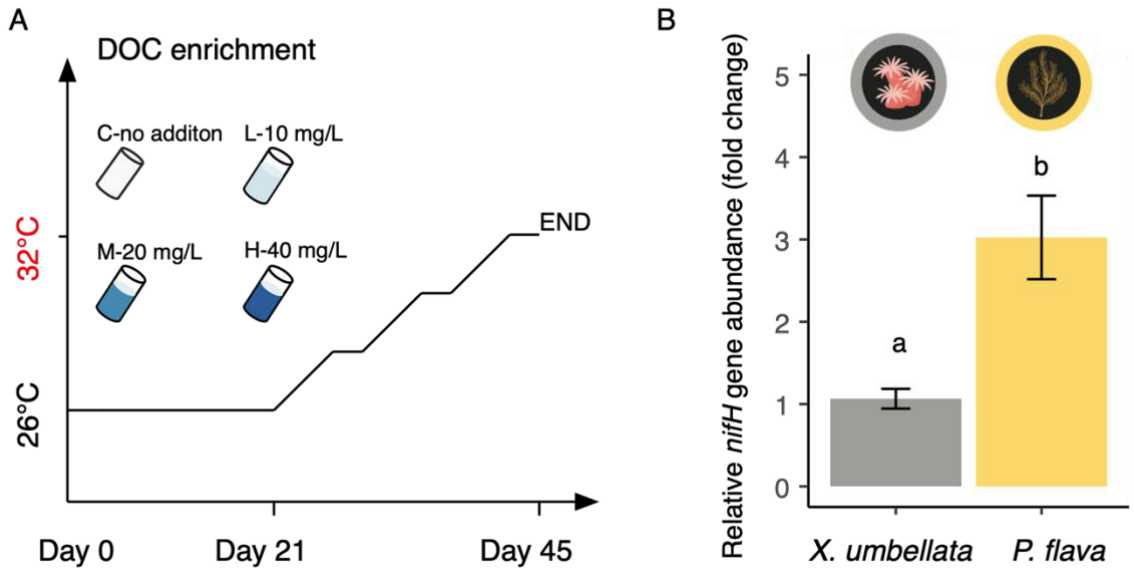

Fig 2.1 Experimental design. A Design of the 45-day aquarium experiment. Three levels of dissolved organic carbon (DOC) enrichment were achieved by daily glucose addition throughout the experiment. A step-wise increase in temperature was applied from day 21 to day 45, with a 2ºC increase every 8 days. **B** The relative abundance of diazotrophs in corals *X. umbellata* and *P. flava* before the start of the experiment.

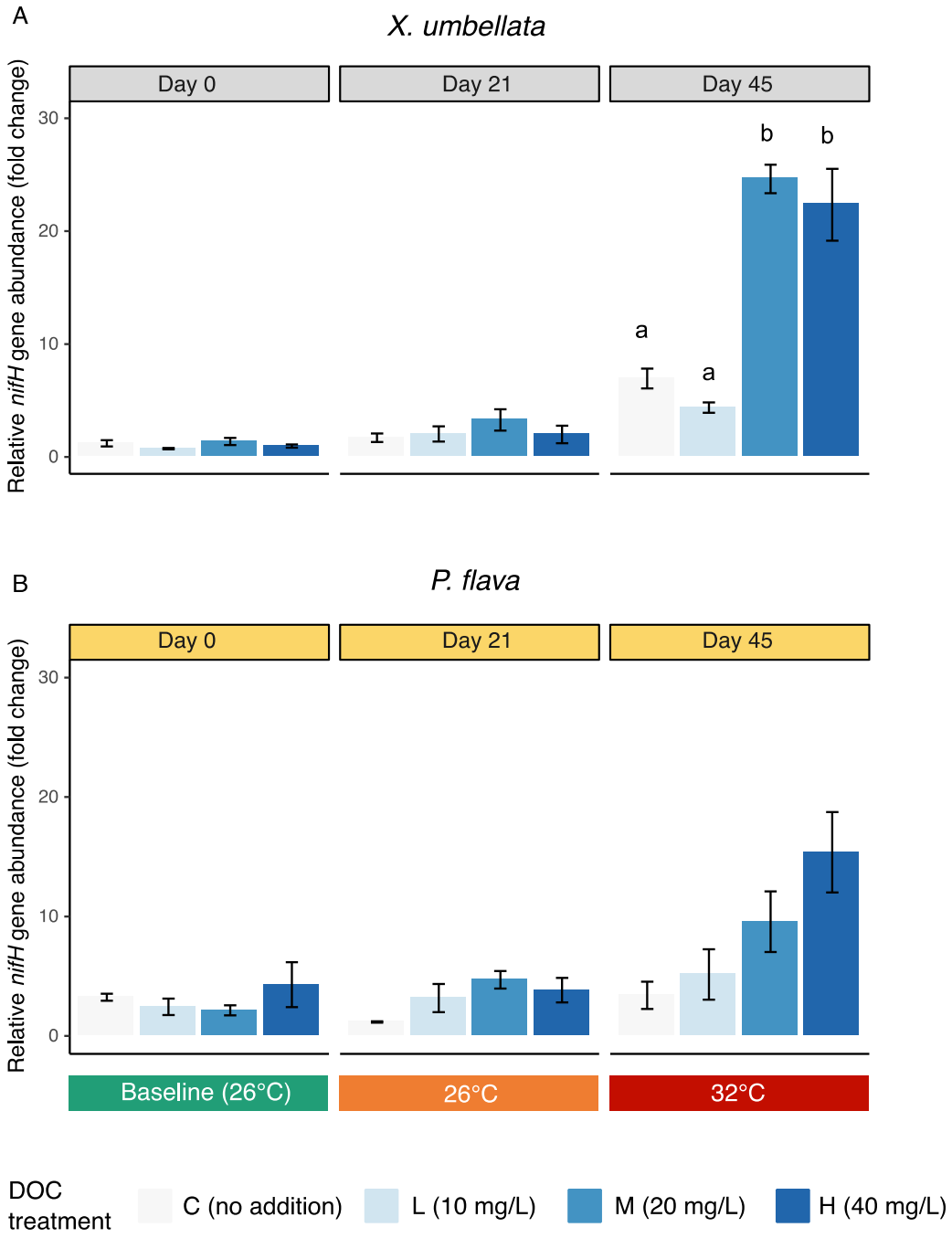

Fig. 2.2 Relative diazotroph abundances in corals A *X. umbellata* and **B** *P. flava* under ambient condition, dissolved organic carbon (DOC) enrichment, and combined DOC enrichment and heat stress. Relative fold change in copy numbers of the *nifH* gene referenced to the 16S rRNA gene and in relation to day 0 control samples of *X. umbellata* (n = 3). All data are presented as mean ± SE (n = 3 fragments each). Different letters above bars indicate significant differences between groups (two-way ANOVA with Tukey HSD, $P < 0.05$).

2.5 Discussion

Diazotrophs support the productivity of corals in oligotrophic waters (4, 5), but may contribute to destabilize the holobiont functioning when overstimulated (13, 14, 19). Currently, studies on cnidaria-associated diazotrophs are largely focused on reef-building corals due to their importance as major ecosystem engineers of tropical coral reefs. Here, we provided the first assessment on the dynamics of diazotroph abundance in two octocoral species under DOC enrichment, and its interactions with heat stress over the course of a 6-week aquarium experiment. We found that DOC enrichment caused no significant effect on the relative abundance of diazotrophs in two investigated octocorals at 26 °C but stimulated their abundances by up to 6 folds in the soft coral *X. umbellata,* and by up to 4 folds in the gorgonian *P. flava* under heat stress (32 °C). Our results suggest that ocean warming may stimulate the effect of DOC enrichment on the proliferation of diazotrophs in octocorals, potentially destabilizing the octocoral holobiont, as discussed below.

2.5.1 Diazotroph abundances in octocorals are host-specific

While host-specific patterns of N_2 fixation rates have been recorded for octocorals, a molecular characterization is currently missing (5, 6). Here, we show the relative abundance of diazotrophs is markedly higher in the autotrophic gorgonian *P. flava* compared to the mixotrophic soft coral *X. umbellata* under an undisturbed condition. This observation aligns with the previously described patterns of diazotroph abundances in reef-building corals (8). This suggests that the mixotrophic *X. umbellata* exhibits a flexible feeding strategy that allows access to various nutrient sources, thereby potentially rendering it less dependent on diazotrophs as reflected in their lower abundances. In contrast, the highly autotrophic *P. flava* may have a stronger dependence on diazotrophs to fulfill the nitrogen requirements for their growth and metabolisms (8, 37, 38).

Host-specific diazotroph abundances may be also linked to the differentiable morphology of two investigated octocorals (38, 39). The *P. flava* holobiont contains an axial skeleton containing calcium carbonate inclusions that may provide a favorable habitat for endolithic bacteria similar to the skeleton of reef-building corals (40), which is absent in *X. umbellata* (41, 42). These endolithic diazotrophs, however, appear to constitute a major component of diazotroph communities in the coral holobiont (6, 43). On the other hand, *X. umbellata* exhibits unique pulsation behaviors that provide numerous advantages to the

holobiont, including enhanced O_2 availability and facilitated nutrient supplementation (39, 44). However, as most diazotrophs are particularly sensitive to the high oxygen or high inorganic nitrogen levels (45), these benefits may instead constrain the diazotroph population in this soft coral holobiont. Taken together, our findings suggest that diazotrophs may be common members of the octocoral microbiome, as suggested previously (22), and that patterns of diazotroph abundances align with the heterotrophic capacity and morphology of their octocoral host.

2.5.2 DOC stimulated diazotroph abundances in heat-stressed octocorals

The relative abundances of diazotrophs in both octocoral species were unaffected by DOC enrichment at 26 °C. This is in contrast with previous finding on reef-building corals where 10 mg L^{-1} DOC enrichment significantly and rapidly promoted their associated diazotroph abundance and activity (13). Notably, octocorals are commonly found in high abundance on degraded coral reefs threatened by anthropogenic change (25, 27). Recent studies have shown that DOC enrichment caused no negative effect on the photosynthetic and respiration rates of octocorals (23), and a similar phenomenon was observed in the symbiotic upside-down jellyfish, *Cassiopea* (46). As such, octocoral holobionts may be better adapted to cope with the environmental DOC enrichment compared to reef-building coral holobionts. At this point, it remains unclear why diazotroph abundances in octocorals are stable during DOC enrichment. Yet, this finding may partially contribute to explain the success of octocorals in disturbed coral reef environments (25).

During heat stress of 32 °C, moderate (20 mg L^{-1}) and high (40 mg L^{-1}) DOC enrichment significantly promoted the relative abundances of *nifH* gene in two octocoral species: the soft coral *X. umbellata* and the gorgonian *P. flava*. Such increases in *nifH* abundance have previously been linked to increases in *nifH* gene expression and N_2 fixation rate and are therefore used as a proxy of diazotroph activity (8). Further, increases in diazotroph abundance and activity have been linked to the increases in nitrogen availability in the holobiont, potentially contributing to the bleaching and symbiotic breakdown of reef-building corals (13). In this light, similar mechanisms may be placed in symbiotic octocoral holobionts. While mixotrophic *X. umbellata* maintained a healthy phenotype in the present study, highly autotrophic *P. flava* bleached during moderate and high DOC enrichments under heat stress (29). At this point, the role of diazotrophs in determining the species-specific bleaching response in our investigated octocorals remains yet to be determined. Nevertheless, the

observed increase in diazotroph abundance in both octocoral species during DOC enrichment and heat stress suggests that the pattern of diazotroph regulation may be similar across cnidarian holobionts with different trophic strategies and lifestyles. However, the effects of stimulated N_2 fixation on holobiont functioning are likely depending on the nutritional status of all holobiont members (13, 14). Taken together, our findings suggest that octocorals exhibit a remarkably higher resistance to DOC enrichment in comparison to reef-building corals. However, in future ocean warming scenarios, DOC enrichment may still stimulate the diazotroph proliferation and thereby increase the nitrogen availability in octocoral holobionts.

2.6 Conclusion

Octocorals constitute a substantial component of benthic reef communities (21) and are likely to become one of the most dominant benthic functional group on coral reefs of the future (28). In contrast to reef-building corals, the abundance of diazotrophs in octocorals appears to be less affected by DOC enrichment. Concomitant with heat stress, however, DOC enrichment can significantly stimulate the abundance of diazotrophs in octocorals. As a result, DOC enrichment and heat stress may jointly affect symbiotic interactions in octocorals, potentially contributing to the destabilization of holobiont nutrient cycling. Therefore, reducing DOC loading from local anthropogenic impacts may effectively help corals and other symbiotic cnidarians cope with the effect of ocean warming.

2.7 Data Accessibility

The Sanger sequencing data derived from *COI*, *mutS*, and 28S rRNA gene PCR amplicons for octocoral identification have been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB43824.

2.8 Author Contributions

NX, CW, and AG designed and conceived the research; CW contributed octocoral samples and aquarium facility; AG contributed analytical reagents for molecular works; CP and NR contributed the PCR protocol for *nifH* gene amplification; NX and AM analyzed samples; NX conducted data visualization and statistical analysis; NX wrote the paper with improvement suggestions from CP, NR, CRV, CW and AG. All authors revised the manuscript and gave final approval for publication.

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2.10 Supplementary materials

2.10.1 Supplementary Method

2.10.1.1 *nifH* positive control

The genome sequence of *Azotobacter vinelandii* DJ (GenBank: CP001157.1), i.e., a strain of N_2-fixing bacteria, was downloaded from the NCBI (47). Eight selected pairs of *nifH* primers (**Tab. S2.1**) were matched against its genome sequence with the in-built function 'Test with Saved Primers' in Geneious Prime (version 2020.1.1). One *nifH* sequence region which *in silico* bound to all primer pairs (**Fig. S2.1**; position 136,759 to 137,154 of genome sequence of *Azotobacter vinelandii* DJ) was verified as a positive control and synthesized at the General Biosystems company (Morrisville, USA).

2.10.1.2 Evaluation of 8 *nifH* primer pairs

The *nifH* gene amplification was tested with eight primer pairs (**Table. S2.1**) using genomic DNA of octocoral samples and our designed positive control as DNA templates. For primer testing, our work flow was: a) screening for the presence of the *nifH* gene with 8 primer pairs usingTaq DNA polymerase Master Mix (VWR, USA) according to the corresponding published cycling condition; b) determining the best annealing temperature for primer pairs with visible bands by gel electrophoresis; c) upon confirmation of a single amplification product using gel electrophoresis, amplicon gel slices from different samples were cut out, and DNA was extracted and purified using the Monarch® DNA Gel Extraction Kit (NEB, USA) following the manufacturer's protocol. The *nifH* amplicon was qualified by Sanger sequencing at StarSeq (Mainz, Germany); d) optimizing PCR using chemical enhancers: $MgCl_2$ (25 mM, VWR, USA) and GC-enhancer (OneTaq® High GC Enhancer, NEB, USA).

2.10.2 Supplementary Results

2.10.2.1 Primer evaluation and *nifH* gene qualification

The 8 published primer pairs chosen for *nifH* gene amplification are located at the front half of the *nifH* gene corresponding to the positive control *Azotobacter vinelandii DJ* (base position 19 - 495) (**Fig. S2.1**). All primer pairs successfully amplified the *nifH* fragments when using the positive control as DNA template. In contrast, only two primer pairs (OF/OR and IGK3/DVV) managed to amplify *nifH* fragments from our investigated octocorals, with a specific *nifH* product only amplified by IGK3/DVV (**Table S2.1**).

2.10.3 Supplementary Table

Table S2.1 The *nifH* gene primers previously used to amplify diazotroph in corals and results of PCR amplifications in corals *X. umbellata* and *P. flava*.

[a] Forward and reverse primers are indicated by the starting letters F and R, respectively. For the degenerate nucleotide codes, M = A/C; R = A/G; W = A/T; S = C/G; Y = C/T; K = G/T; V = A/C/G; H = A/C/T; D = A/G/T; B = C/G/T; N = A/C/G/T.

[b] Abbreviations for coral species are as follows: *Isopora palifera* (Ip); *Galaxea astreata* (Ga); *Pavona decussata* (Pd); *Porites lutea* (Pl); *Pleuractis granulosa* (Pg); *Ctenactis echinata* (Ce); *Pocillopora verrucosa* (Pv); *Stylophora pistillata* (Sp); *Montipora capitata* (Mc); *Montipora flabellata* (Mf); *Montastraea cavernosa* (Mcs); *Oculina patagonica* (Op).

[c] "-" means unamplified PCR product but valid positive control; "+" means amplified target PCR product; "NS" means non-specific product.

Primer (5'-3')[a]	Product size	Coral species	Coral reference	This study[b]
F:nifH1(TGYGAYCCNAARGCNGA); R:nifH2(ANDGCCATCATYTCNCC)	460 bp	*Ip*	[43]	–
F:PolF(TGCGAYCCSAARGCBGACTC); R:PolR(ATSGCCATCATYTCRCCGGA)	360 bp	*Ga; Pd; Pl*	[48]	–
F:F2(TGYGAYCCIAAIGCIGA); R:R6(TCIGGIGARATGATGGC)	359 bp	*Pg*; *Ce*; *Pv*; *Sp*	[8,13,49,50]	–
F:OF(ATGTCGGYTGYGAYCCSAARGC); R:OR(ATGGTGTTGGCGGCRTAVAKSGCCATCAT)	389 bp	*Mc; Mf*	[51]	NS
F:IGK(AARGGNGGNATHGGNAA); R:YAA(ATRTTRTTNGCNGCRTA)	464 bp	*Mc*	[52]	–
F:IGK3(GCIWTHTAYGGIAARGGIGGIATHGGIAA); R:DVV(ATIGCRAAICCICCRCAIACIACRTC)	383 bp	*Sp*	[9, 16, 20, 53]	+
F:mnifH-F(TGYGAYCCNAARGCNGA); R:mnifH-R(ADNGCCATCATYTCNCC)	360 bp	*Op*	[19]	–
Nest PCR F:IGK(AARGGN GGNATHGGNAA); R:YAA(ATRTTRTTNGCNGCRTA); F:nifH1(TGYGAYCCNAARGCNGA); R: nifH2(ANDGCCATCATYTCNCC)	359 bp	*Mcs*	[52]	–

2.10.4 Supplementary Figure

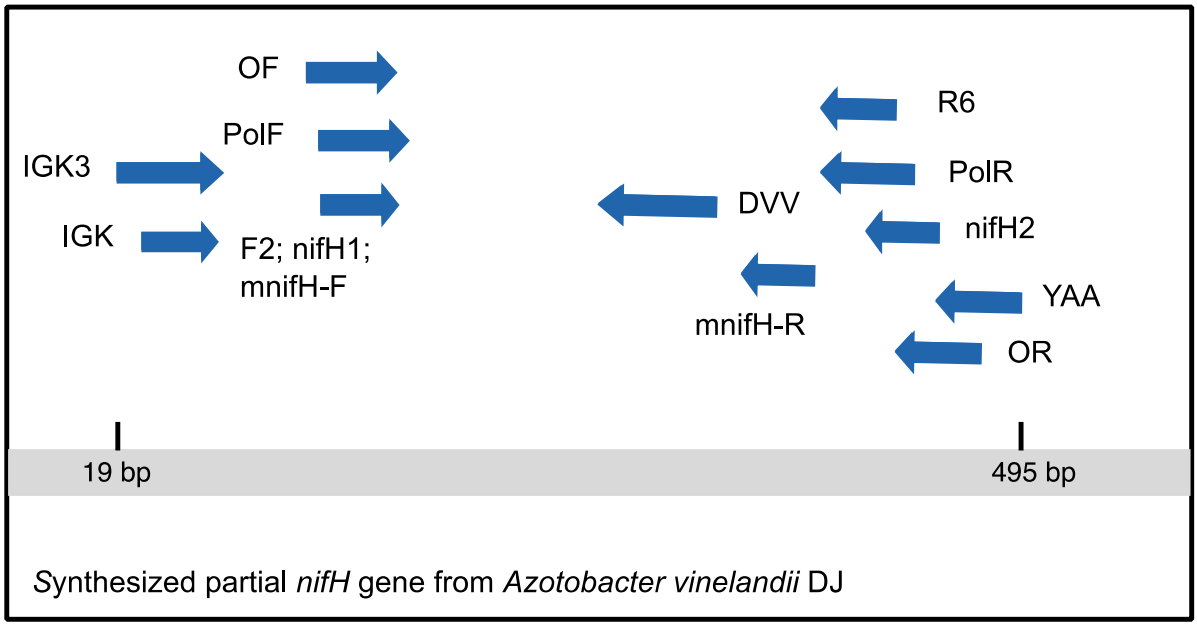

Fig. S2.1 Diagram showing binding positions of selected primers to amplify the N_2 fixation *nifH* gene. The positions for *nifH* primers are correlated with the *nifH* sequence from a diazotrophic strain *Azotobacter vinelandii* DJ (GenBank: CP001157.1).

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Chapter 3 Contrasting microbiome dynamics of putative denitrifying bacteria in two octocoral species exposed to dissolved organic carbon (DOC) and warming

Contrasting microbiome dynamics of putative denitrifying bacteria in two octocoral species exposed to dissolved organic carbon (DOC) and warming

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Running Head: Octocoral Microbial Community and Denitrification

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3.1 Abstract

Mutualistic nutrient cycling in the coral-algal symbiosis depends on limited nitrogen (N) availability for algal symbionts. Denitrifying prokaryotes capable of reducing nitrate or nitrite to dinitrogen could thus support coral holobiont functioning by limiting N availability. Octocorals show some of the highest denitrification rates among reef organisms, however little is known about the community structures of associated denitrifiers and their response to environmental fluctuations. Combining 16S rRNA gene amplicon sequencing with *nirS* in-silico PCR and quantitative PCR, we found differences in bacterial community dynamics between two octocorals exposed to excess dissolved organic carbon (DOC) and concomitant warming. While bacterial communities of the gorgonian *Pinnigorgia flava* remained largely unaffected by DOC and warming, the soft coral *Xenia umbellata* exhibited a pronounced shift towards *Alphaproteobacteria* dominance under excess DOC. Likewise, the relative abundance of denitrifiers was not altered in *P. flava*, but decreased by one order of magnitude in *X. umbellata* under excess DOC likely due to decreased proportions of *Ruegeria* spp. Given that holobiont C:N ratios remained stable in *P. flava* but showed a pronounced increase with excess DOC in *X. umbellata* host, our results suggest that microbial community dynamics may reflect the nutritional status of the holobiont. Hence, denitrifier abundance may be directly linked to N availability. This suggests a passive regulation of N cycling microbes, which could help stabilize nutrient limitation in the coral-algal symbiosis and thereby support holobiont functioning in a changing environment.

3.2 Introduction

Coral reefs are hotspots of marine biodiversity and primary productivity in oligotrophic tropical oceans (1). Corals, the ecosystem engineers of these reefs, are key to supporting these ecosystems (2). The symbiosis with intracellular dinoflagellate algae of the family Symbiodiniaceae is central to this ecological success as it enables corals access to heterotrophic as well as autotrophic nutrient sources to supporting growth and productivity (3, 4). In particular, the translocation of organic carbon (C) in the form of photosynthates by symbiotic algae is a major energy source for the coral host, which provides inorganic nutrients and carbon dioxide from its catabolism to supporting algal photosynthesis (5). However, the efficient symbiotic trade of C in the coral-algal symbiosis depends heavily on limited nitrogen (N) availability for the algae. Constant N limitation of algal symbionts is required to limit their populations and ensure the accumulation of excess photosynthates available for translocation (5, 6). Given the pronounced environmental fluctuations and seasonality in coral reefs, the functioning of symbiosis thus depends on active and/or passive regulation of nutrient availability for algal symbionts, summarized in (7). Importantly, the nutrient availability in the symbiosis does not depend on interactions of the host and its symbiotic algae alone (8, 9). Corals also associate with a diverse prokaryotic microbiome with varying degrees of taxonomic flexibility depending on host species and environmental conditions (10). Many members of the prokaryotic microbiome are or may be actively involved in the provision and recycling of limiting nutrients such as N, Phosphorus (P), thereby altering nutrient availability for the holobiont (11-14). As such, the ecological success of corals likely depends on an intricate functional interplay of all its microbial associates. This diverse multi-species assemblage termed the coral holobiont extends the metabolic properties of its members and may help their rapid adaptation to changing environmental conditions (15).

For millions of years, the functional interplay between holobiont members has formed the basis of the ecological success of corals and the reefs they support (16-18). Yet in recent decades, anthropogenic activities have led to widespread coral mortality and reef degradation (19, 20). Global and local stressors such as ocean warming and labile DOC loading are known to disrupt holobiont functioning, resulting in coral bleaching, i.e., the collapse of the coral-algal symbiosis, or coral diseases (21-23). The breakdown of this symbiosis is not exclusively restricted to interactions between the coral host and their algal symbionts, but involves other members of the holobiont as well, e.g., prokaryotes (10, 24, 25). Given that the stability of the coral-algal symbiosis is dependent on N limitation of the algal symbionts, microbial N cycling

may stabilize or destabilize holobiont functioning depending on the environmental conditions (7, 14, 26).

In particular, denitrifiers, i.e., prokaryotes that encompass the reduction of nitrate or nitrite to dinitrogen gas (27), might help alleviate excess N stress of holobionts (7). Denitrifiers appear to be widely associated with many reef organisms and their activity was recently confirmed in corals from the Red Sea (28). Specifically, it has been observed that the activity of microbial denitrification in coral holobionts increased with environmental N levels (29). As such, changes in community structure, abundance and activity of denitrifiers might directly affect coral holobiont functioning by altering N availability for other holobiont members. Yet, it remains unexplored that how environmental change such as ocean warming and excess DOC influences coral-associated denitrifying bacterial communities.

To date, most studies have focused on the bacterial community dynamics of scleractinian corals given their importance as reef ecosystem engineers (25, 30). In contrast, even though octocorals constitute highly abundant members of benthic communities of coral reefs, their bacterial community dynamics under environmental stress remain largely unexplored (31). Octocorals were recently shown to exhibit some of the highest denitrification rates among Red Sea reef organisms and substrates (32), indicating a potential importance of this microbial functional trait to octocoral health and holobiont functioning. However, the (a)biotic drivers of denitrifier abundance and community composition in octocoral holobionts are unknown.

Here, we set out to explore the effects of excess DOC and concomitant warming on bacterial community structure with a focus on diversity and abundance of putative denitrifiers associated with the pulsating soft coral *Xenia umbellata* (Lamarck, 1816) and the gorgonian *Pinnigorgia flava* (Nutting, 1910). The experiment consisted of two consecutive phases. In phase one, excess DOC enrichment was performed over the course of 21 days at varying concentrations via administration of daily glucose dosing. In phase two, the continued DOC enrichment was combined with step-wise warming for 24 days (**Fig. 3.1**). By doing so, we aimed to: first, provide a comprehensive overview of the bacterial symbionts associated with two common and taxonomically distinct octocoral species and their response to excess DOC and ocean warming; and second, to provide a comparative assessment on the abundance,

diversity, and community dynamics of associated putative denitrifiers in these octocoral holobionts.

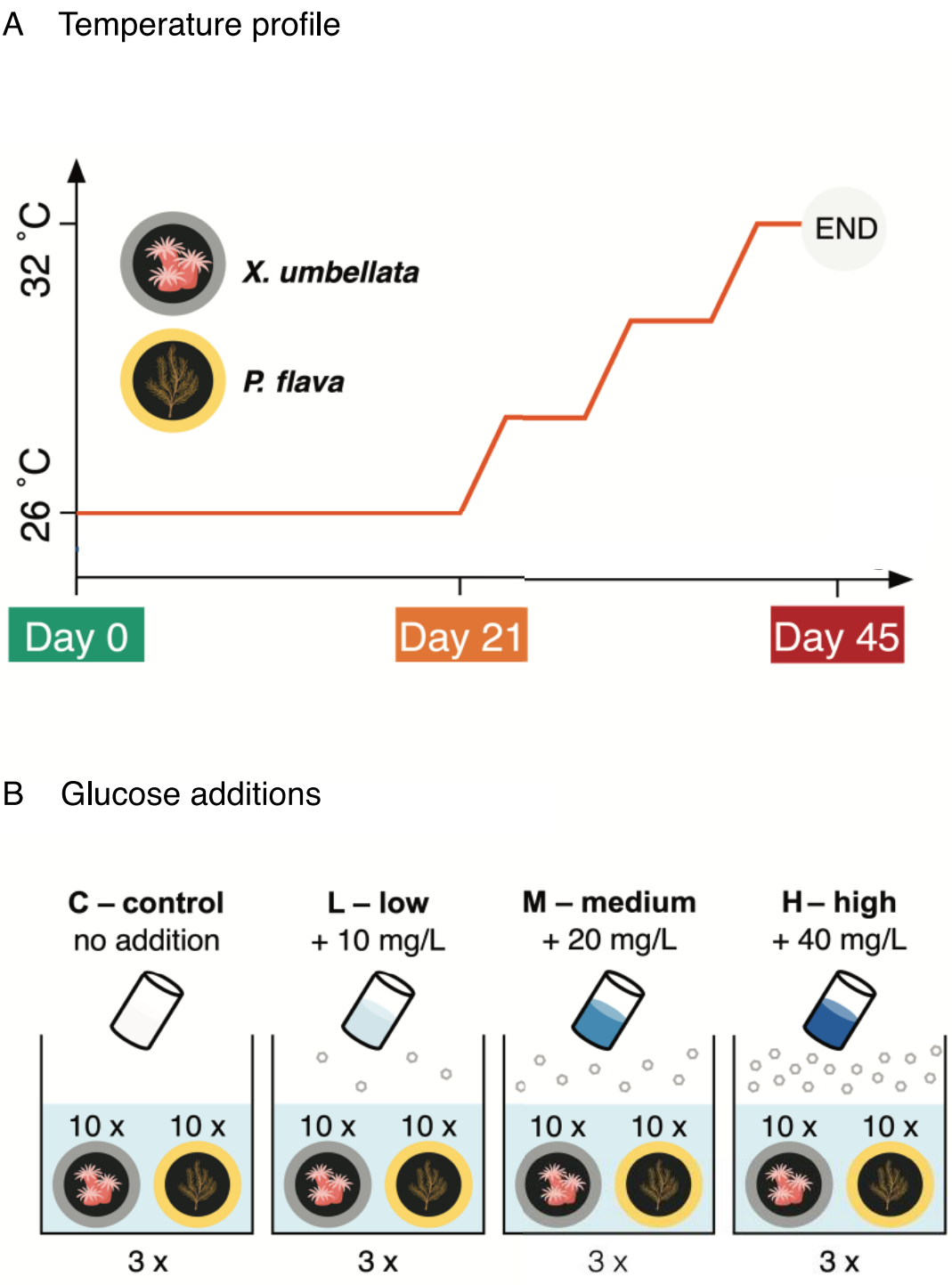

Fig. 3.1 Design of the conducted manipulative aquarium experiment. A Step-wise increases in temperature started from day 21 to day 45 with an increase in 2°C every 8 days. B Dissolved organic carbon (i.e., glucose) additions were applied daily throughout the experiment.

3.3 Materials and Methods

3.3.1 Coral preparation and maintenance

The soft coral *X. umbellata* and the gorgonian *P. flava* were cultivated (temperature: 26 ± 0.5 °C; pH 7.8 ± 0.2; salinity: 35 ± 3 ‰) for more than 2 years at the Marine Ecology Department of the University of Bremen. Coral species were identified by barcoding gene (*COI*, *mutS* and 28S rRNA gene) amplification and sanger sequencing. Small *X. umbellata* fragments (1-2 cm in side length) were cut from 5 mother colonies (5×7×12 cm) and fixed on cubical-shaped calcium carbonate coral holders (1×1 cm) with rubber bands. After fragments recovered from fragmentation for 7 days and attached to the holders, rubber bands were removed. Simultaneously, branches of 3 - 4 cm in height were cut from 4 mother colonies (18×1×24 cm) of *P. flava,* and subsequently attached to coral holders using aquarium moss coral fix glue. Thereafter, 120 fragments of *X. umbellata* and 120 fragments of *P. flava* were distributed among 12 experimental aquaria tanks (water volume 50 L) and acclimated for 10 days prior to the experiment. Each aquarium was equipped with a thermostat (3613 aquarium heater, 75 W 220-240 V), a pump (EHEIM Compact On 300 pump) and a protein skimmer that were all purchased from EHEIM GmbH and Co. KG in Germany to maintain stable aquarium conditions. Additionally, LED lights (Royal Blue-matrix module and Ultra Blue White 1:1-matrix module, WALTRAt day time® LED light, Germany) were used to simulate day - night rhythm of 12 h-12 h at the intensity of 120.8 ± 10.2 µmol quanta m^{-2} s^{-1}. No additional feeding was provided and 10% of the artificial seawater was exchanged daily to maintain stable water parameters. To verify stable environmental conditions, salinity (C: 35.39 ± 0.05; L: 35.38 ± 0.08; M: 35.50 ± 0.08; H: 35.11 ± 0.09) and pH (C: 8.15 ± 0.01; L: 8.05 ± 0.02; M: 8.05 ± 0.02; H: 8.03 ± 0.02) were monitored throughout the experiment (**Fig. S3.4**).

3.3.2 Experimental design, sampling, and DNA extraction

In the first phase of experiment, temperature was kept constant at 26 °C for 21 days (**Fig. 3.1**). Daily additions of glucose to the aquaria based on a stock solution (D-Glucose, 40 mg mL^{-1}) were used to simulate four different glucose enrichment levels: control (no addition), low (equivalent to 10 mg L^{-1}), medium (equivalent to 20 mg L^{-1}) and high (equivalent to 40 mg L^{-1}). Daily measurements of total organic carbon (TOC) using a TOC-L analyzer (Shimadzu, Japan) were used to approximate glucose levels in the aquaria and the daily dosing of glucose was adjusted accordingly to achieve the desired enrichment levels. For the second phase, temperature was increased gradually in all aquaria, ultimately adding 2 °C every 8 days to a

final temperature of 32 °C. The range of 26 ºC to 32 ºC represents a latitudinal gradient of mean maximum temperature from north to south across the Red Sea, where *X. umbellata* is widely abundant (33). The temperature maximum of 32 ºC is also close to the thermal physiological limit of this species (34). During this gradual ramping phase, all DOC treatments were continued as described above (**Fig. 3.1**). The experiment was terminated at day 45.

Corals for molecular analysis were collected at day 0 (before DOC additions, as baseline data), after the first phase of the experiment (day 21, DOC treatments at 26 ºC), and after the second phase (day 45, DOC treatments at 32 ºC). For this, fresh coral samples were collected and immediately frozen in liquid nitrogen and stored at -80 ºC until further processing. Additionally, samples for physiological measurements and aquarium seawater inorganic nutrients were collected at day 45 as outlined below. Frozen coral samples were ground into powder over liquid nitrogen using mortar and pestle. Genomic DNA was extracted according to the instruction of Quick-DNA Universal Kit Quick Protocol for Solid Tissue (ZYMO RESEARCH, USA). Afterwards, DNA was quantified by spectrophotometry at 260 nm and 280 nm (Tecan Infinite 200 PRO, Austria) and quality-checked by 1% (wt/vol) agarose gel electrophoresis (Biometra Horizon 58, Germany).

3.3.3 MiSeq 16S rRNA gene sequencing and sequence analysis

The 16S rRNA gene amplicon sequencing was conducted at LGC genomics (Berlin, Germany). The hypervariable regions V3-V4 of the bacterial 16S rRNA gene were amplified and sequenced using the primer pair S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (35). The PCRs included 1-10 ng of DNA extract (total volume 1µl), 15 pmol of each forward primer and reverse primer in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline GmbH, Germany) and 2 µl of BioStabII PCR Enhancer (Sigma-Aldrich Co., Germany). For each sample, the forward and reverse primers had the same 10-nt barcode sequence for multiplexing. PCRs were carried out for 35 cycles using the following parameters: 1 min 96 °C pre-denaturation; 96 °C denaturation for 15 s, 55 °C annealing for 30 s, 70 °C extension for 90 s, hold at 8 °C. The Illumina library was pooled and size selected by preparative gel electrophoresis, and the sequencing was conducted on the Illumina MiSeq platform in a 2×300 bp paired-end run using V3 Chemistry. After demultiplexing and removal of primer sequences from the raw paired-end reads by LGC genomics, further sequence processing was performed according to the DADA2 (1.14.1) pipeline for the generation of exact amplicon

sequence variants (ASVs) (36). Specifically, sequences were filtered and quality trimmed to 225 bp (forward) and 235 bp (reverse) at a maximum expected error rate of 5. Trimmed sequences were pooled and used for error learning and denoising. In total, 77 samples were pooled with 3,858,215 reads in 1,264,025 unique sequences. Denoised sequences were merged, followed by Chimeras removal according to default parameters. A total of 26,902 chimeras were identified out of 40,839 ASVs, singletons generated during the merging step were removed. ASVs between 400 to 430 bp were retained and taxonomically classified by "assignTaxonomy" based on the SILVA database release 138 (37). ASVs that matched chloroplast and mitochondrial sequences were removed prior to further analysis.

An *in silico* PCR for the nitrite reductase *nirS* primer pair, nirS-1F (5'-CCTAYTGGCCGCCRCART-3') and nirS-qR (5'-TCCMAGCCRCCRTCRTGCAG-3') (38) was used to characterize the putative denitrifier community. The program ecoPCR (39) from OBI tools 1.01.22 was launched against the Ensembl Bacteria release 42 with a maximum 3 mismatches and a zero mismatch zone of 2 bp at the 3' end of each primer, retaining fragments between 50 bp and 500 bp. Resultant sequences from ecoPCR with the fragment size between 224 - 227 bp were blasted against the GenBank Nucleotide database (NCBI nucleotide BLAST, date accessed 2020/04/05), and sequences that were not identified as originating from denitrifying *nirS* were removed. The genus affiliation of the remaining *nirS* fragments was used as potential denitrifying taxa to recover denitrifier communities based on our 16S rRNA gene sequencing results. In addition, ASV sequences affiliated to the predominant genus were aligned to SILVA 138, and the accession numbers of nearest relatives from SILVA Incremental Aligner (SINA) output were used to obtain a higher resolved taxonomic path.

3.3.4 Quantification real-time PCR (qPCR) of denitrifying nirS *gene*

We assessed the denitrification potential in the coral holobiont via the relative quantification of *nirS* gene, which catalyzes the conversion of nitrite to nitric oxide in the denitrification cascade (40) and has been previously used to determine denitrifier abundance and diversity (27, 38, 41). The *nirS* gene was amplified using the same primer pair nirS-1F, nirS-qR (38) previously used for *in silico* PCR as outlined above, and validated with Sanger sequencing (StarSEQ, Mainz, Germany). The relative quantification of *nirS* gene abundance was done by qPCR using the CFX96™ Touch Real-Time PCR Detection System (BIO-RAD, USA) by SensiFAST™ SYBR® No-ROX Kit (Bioline, USA). C_T values of *nirS* gene amplicons (as a proxy of denitrifier abundance) were referenced against C_T values of 16S rRNA

gene amplicons (as a reference for total bacterial abundance) using the primer-pair Bact-16S_784F: 5'-AGGATTAGATACCCTGGTA-3' and Bact-16S_1061R: 5'-CRRCACGAGCTGACGAC-3' (42), according to the delta-delta Ct method ($2^{-\Delta\Delta Ct)}$ (43). 10-20 ng of DNA extract was used for 16S rRNA gene and *nirS* qPCRs. Final cycling conditions consisted of a hot-start activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C (*nirS* gene in *X. umbellata*) or 60 °C (*nirS* gene in *P. flava* and 16S rRNA gene for both species) for 20 s, and extension at 72 °C for 30 s. Final extension was carried out at 72 °C for 10 s followed by a melting curve from 65 to 95 °C with increase of 0.5 °C steps every 5 s. The qPCR efficiency was validated by calibration curves of genomic DNA from *E. coli* (ATCC 25922) targeting 16S rRNA gene and DSM 428 *Alcaligenes eutropus H16* targeting *nirS* gene separately.

3.3.5 Seawater inorganic nutrient and coral elemental analysis

Nutrient samples were collected in triplicates at the end of the experiment (i.e., day 45). 50 mL of aquaria seawater was collected through 0.45 µm filters in 50 mL sterilized centrifuge tubes, and immediately frozen at -20 °C until further analysis. Nutrient levels were measured spectrophotometrically using the Infinite 200 PRO (Tecan Infinite 200 PRO, Austria) according to (44). Coral samples for elemental and isotope analysis were collected at day 45. Fresh coral fragments were immediately frozen in liquid nitrogen and stored at -80 °C until further processing.

Corals were thawed at room temperature and homogenized for 30 s at 3,500 rpm with an Ultra Turrax (IKA, Germany). The resulting homogenized coral slurry was separated into coral tissue and algal symbiont fractions by centrifugation at 3,000 g for 5 min (Eppendorf, Germany). The host fraction, i.e. the resulting supernatant was carefully removed by pipetting without disturbing the algal symbiont pellet. Algal symbiont pellets were resuspended in 0.22 µm filtered seawater (FSW) in sterilize 2 mL Eppendorf tubes, and host and algal samples were dried at 40 °C for one week. After the dried matter was pulverized using clean mini pestles; 1.0 mg of coral tissue or algal symbiont sample was used for measuring total nitrogen content (TN). Further, 1.0 mg of coral tissue or algal symbiont sample mixed with 200 µL 1 mol L^{-1} HCL was used for analyzing total organic carbon content (TCorg). TN and TCorg were analyzed in an elemental analyzer (Euro EA, Germany), Isotopic $\delta^{15}N$ and $\delta^{13}C$ signatures were measured using an Isotope Ratio MS (Thermo Fisher, USA), following (45).

3.3.6 Statistical analysis

All statistical analyses were performed in R studio 3.6.1, specifically *vegan* for multivariate statistics (46) and *ggplot2* for visualization (47). Alpha diversity indices were calculated based on repeated (n = 100) random subsampling of ASVs to the minimum library size at sequencing depth 3,000. Data normality was determined by the Shapiro–Wilk test, and statistical differences between different timepoints and DOC treatments for each species was tested using two-way analysis of variance (ANOVA) with Tukey's HSD as a post-hoc comparison.

Beta dispersion of samples was conducted for each coral species using function "betadisper". Beta diversity was evaluated between species, timepoints and DOC treatments by non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarities of relative ASV proportions. Analysis of similarities (ANOSIM) was used to illustrate the dissimilarity of bacterial community structures between two coral species at day 0. To illustrate the significant difference in bacterial variations at ASV level from different timepoints and DOC treatments, permutational multivariate analysis of variance (PERMANOVA, 'adonis' function, 999 permutations) based on Bray-Curtis dissimilarity was applied along with ANOSIM as post-hoc comparisons. An ANOSIM R value close to 1 suggests a strong separation between groups, while values close to 0 indicates an overlap between groups (48). After log transformation to meet data normality, homogeneity of variance and independence, the qPCR data was analyzed by two-way ANOVA and Tukey's HSD. Seawater inorganic nutrient levels in aquaria and elemental changes in animal host and algal symbionts respectively were analyzed by one-way ANOVA and Tukey's HSD.

3.4 Results

3.4.1 Overview of 16S rRNA gene sequencing data

Over the duration of 45 days of the aquarium experiment with two octocoral species, using 4 levels of DOC concentrations and 2 different temperatures, a total of 78 coral fragments were collected for 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform. Of these, 76 samples passed quality control, resulting in 8,145 amplicon sequence variants (ASVs) based on 1,150,262 sequences. Of total ASVs, 5,703 ASVs were present in both coral species. 1,847 ASVs were exclusively associated with the soft coral *X. umbellata* and 595 ASVs with

the gorgonian *P. flava* only. Under undisturbed condition, the total number of bacterial ASVs associated with *X. umbellata* (6,344 ASVs at day 0) was higher than in *P. flava* (4,260 ASVs at day 0). However, over the course of our experiment, *X. umbellata* showed a decline in total ASV diversity regardless of DOC treatments (1,618 and 1,058 ASVs for day 21 and 45, respectively), as well as significantly reduced Inverse Simpson Index (**Fig. S3.2**; Two-way ANOVA, Time-effect: $F_{2,24} = 14.97$, $P < 0.001$, DOC-effect: $F_{4,24} = 5.76$, $P = 0.002$). Specifically, compared to day 0, the Inverse Simpson Index experienced an 80% reduction at day 21 (**Fig. S3.1; Fig. S3.2;** Tukey's HSD, $P = 0.003$) and an 84% reduction at day 45 (Tukey's HSD, $P < 0.001$). In contrast, ASV numbers and Inverse Simpson Index remained stable in the *P. flava* bacterial microbiome (**Fig. S3.1; Fig. S3.2;** Two-way ANOVA, Time-effect: $F_{2,26} = 1.57$, $P = 0.227$, DOC-effect: $F_{4,26} = 0.20$, $P = 0.936$) throughout the experiment.

3.4.2 Distinct bacterial communities and bacterial community responses of *X. umbellata* and *P. flava*

At day 0, i.e. just prior to the start of the experiment, both octocoral species were associated with distinct dominant bacterial taxa (**Fig. 3.2B; Fig. 3.2C**; ANOSIM, $R = 0.68$, $P = 0.001$). In *X. umbellata*-associated bacterial communities, *Alphaproteobacteria* (25-30% proportion; ca. 70% of sequences affiliated to *Rhodobacteraceae*), *Bacteroidia* (20-25% proportion; ca. 75% of sequences affiliated to *Kordia*) and *Gammaproteobacteria* (15-25% proportion; ca. 70% of sequences affiliated to *Alteromonadaceae*) were identified as dominant classes. In contrast, *P. flava* was dominated by *Alphaproteobacteria* (20-40% proportion; ca. 65% of sequences affiliated to *Rhodobacteraceae*), *Gammaproteobacteria* (10-40% proportion; ca. 16% of sequences affiliated to *Vibrionaceae*) and *Campylobacteria* (15-80% proportion; ca. 70% of sequences affiliated to *Arcobacteraceae*) (**Fig. 3.2C**).

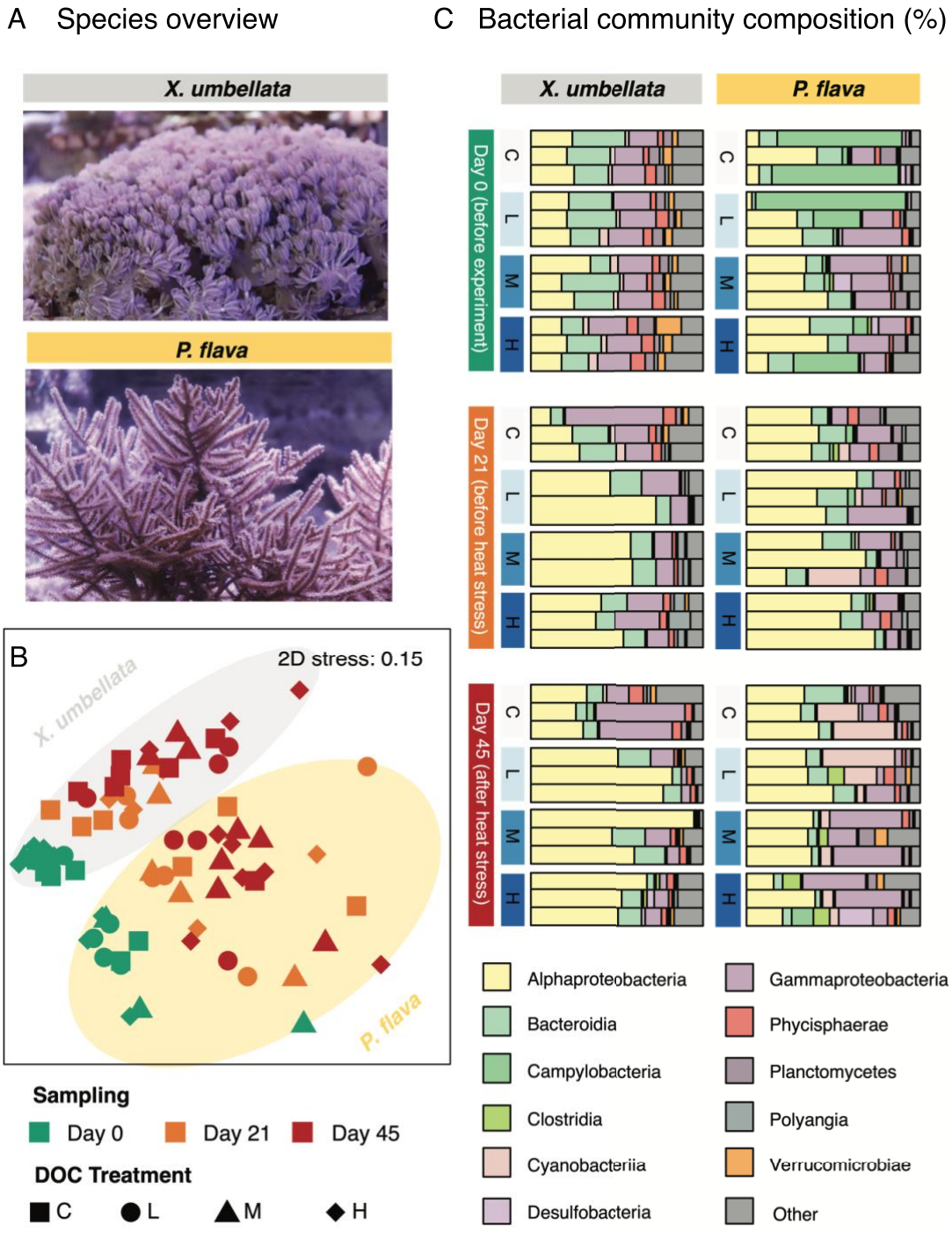

Fig. 3.2 Bacterial community compositions of corals *X. umbellata* and *P. flava* over the course of the experiment. A Representative photographs of octocorals. **B** Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity matrix of bacterial community compositions associated with coral samples at day 0, day 21, and day 45. **C** Stacked bar plots of bacterial community compositions of corals across different timepoints and DOC treatments. Stacked bar plots display the 10 most abundant bacterial classes (>1%, determined for each coral species separately).

X. umbellata bacterial communities significantly varied across timepoints (**Fig. 3.2C;** PERMANOVA, $F_{2,22}$ = 5.07, P = 0.001), and were significantly affected by excess DOC (PERMANOVA, $F_{3,22}$ = 1.92, P = 0.003). At day 21, bacterial community structure of *X. umbellata* at control did not markedly change from day 0 (**Fig. 3.2C**; ANOSIM, R = 0.52, P = 0.132). Yet, bacterial communities under excess DOC separated well from the control (**Fig. 3C**; ANOSIM, R = 1.00, P = 0.132) by showing a two-fold increase in the proportion of *Alphaproteobacteria*, primarily ASVs affiliated to *Rhodobacteraceae* (63-75% proportion of *Alphaproteobacteria*) and *Hyphomonadaceae* (25-28%). Until day 45, following continuous excess DOC paired with a temperature increase to 32 °C, *X. umbellata* bacterial communities were dominated by *Alphaproteobacteria* (mainly *Rhodobacteraceae*; 75-80% of all sequences in this class), a higher proportion than in corals with excess DOC at day 21 (**Fig. 3C**; ANOSIM, R = 1.00, P = 0.132). *P. flava*-associated bacterial communities differed significantly over time (PERMANOVA, $F_{2,24}$ = 2.60, P = 0.001). This was first and foremost driven by the 8-fold increased proportion of ASV5, which was affiliated with *Paraspirulinaceae*, in corals without excess DOC. In contrast, the bacterial community structure of *P. flava* did not significantly respond to excess DOC throughout the experiment (**Fig. 3C**; PERMANOVA, $F_{3,24}$ = 0.99, P = 0.500).

3.4.3 Pronounced effects of excess DOC on composition and abundance of putative denitrifiers associated with *X. umbellata*

The *nirS* in-silico PCR and 16S rRNA gene sequencing retrieved a total of 97 ASVs distributed over 14 putative denitrifying genera from 76 octocoral samples. Putative denitrifying taxa were represented by 12 genera and 75 ASVs and accounted for approximately 10% of the *X. umbellata* bacterial community. The putative denitrifying community in *P. flava* varied more across samples than in *X. umbellata*, with denitrifiers being represented by 14 genera from 79 ASVs, accounting for up to 23% of the overall bacterial community. *Ruegeria* (ca. 90% of denitrifier-affiliated sequences, across 23 ASVs dominated by 60-65% ASV8 and 26-35% ASV11) showed its dominance in both octocoral denitrifying communities at day 0. *Labrenzia* (incl. ASV43, ASV126 and ASV179) was the second most dominant genus and occupied a higher proportion in *P. flava* (**Fig. 3.3A**). The denitrifying community of *X. umbellata* revealed a ca. 80% reduction in sequence proportion related to *Ruegeria* spp. and a ca. 30% increase in the proportion of sequences affiliated with *Dinoroseobacter* spp. after 21 days of excess DOC (**Fig. 3.3A**). However, this shift did not affect the cumulative relative abundance of putative denitrifiers, as estimated by *nirS* gene relative abundance (**Fig. 3.3B;**

ANOVA, $F_{3,24}$ = 2.280, P = 0.105). Denitrifying community structure in *P. flava* showed no significant response to DOC, as well as a non-significant change in the *nirS* relative abundance based on qPCR (**Fig. 3.3B**; ANOVA, $F_{3,24}$ = 0.961, P = 0.427).

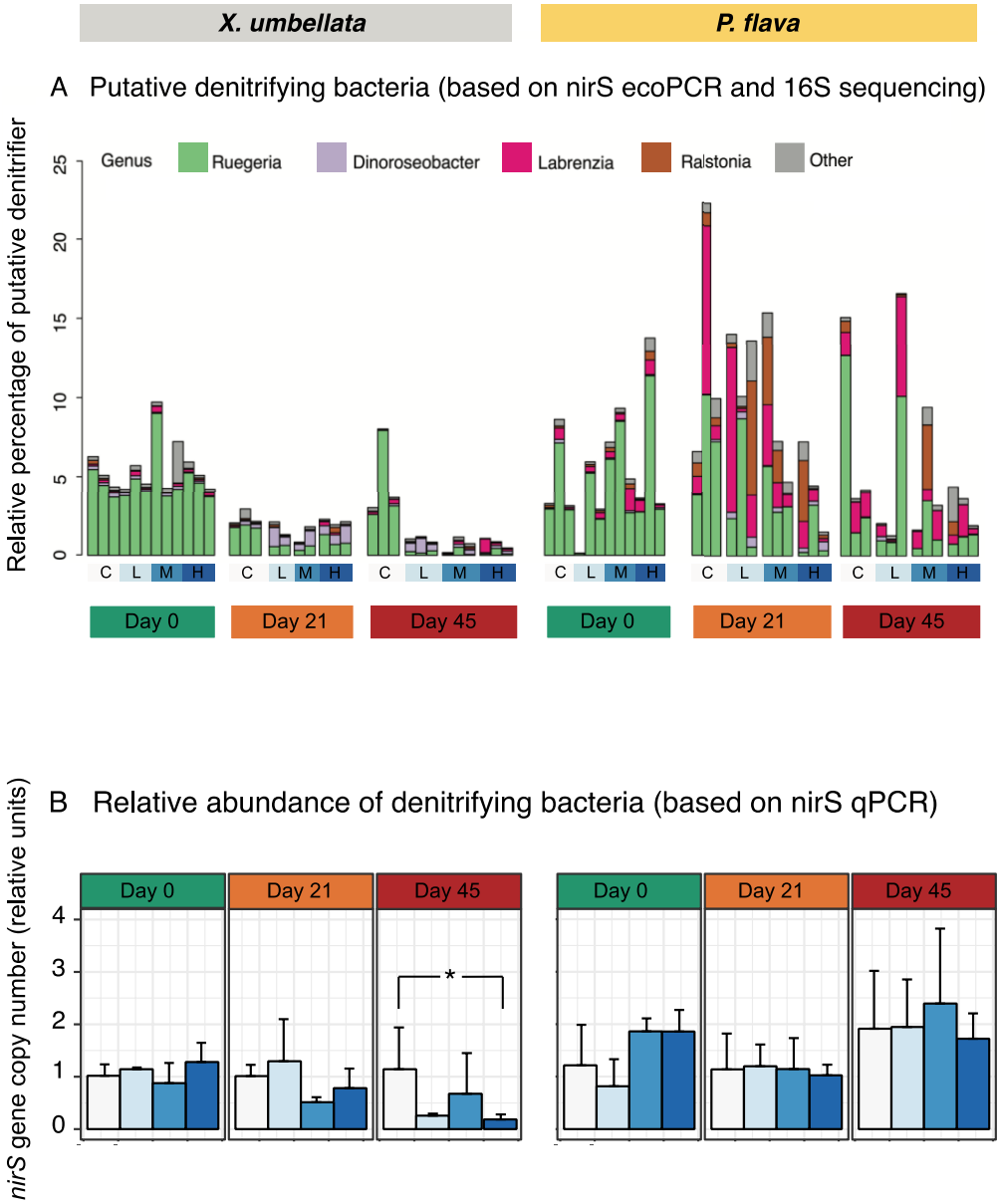

Fig. 3.3 Community compositions and relative abundances of putative denitrifiers in corals *X. umbellata* and *P. flava* over the course of the experiment. A Relative proportions of denitrifier genera of corals *X. umbellata* and *P. flava* inferred by *nirS* in-silico PCR in relation to the total bacterial community from 16S rRNA gene sequencing. **B** Relative fold changes in copy numbers of *nirS* gene referenced to 16S rRNA gene and in relation to the day 0 control samples (n = 3) of corals *X. umbellata* and *P. flava*. Values are means ± SD, and the asterisk indicates statistically significant differences (*$p < 0.05$).

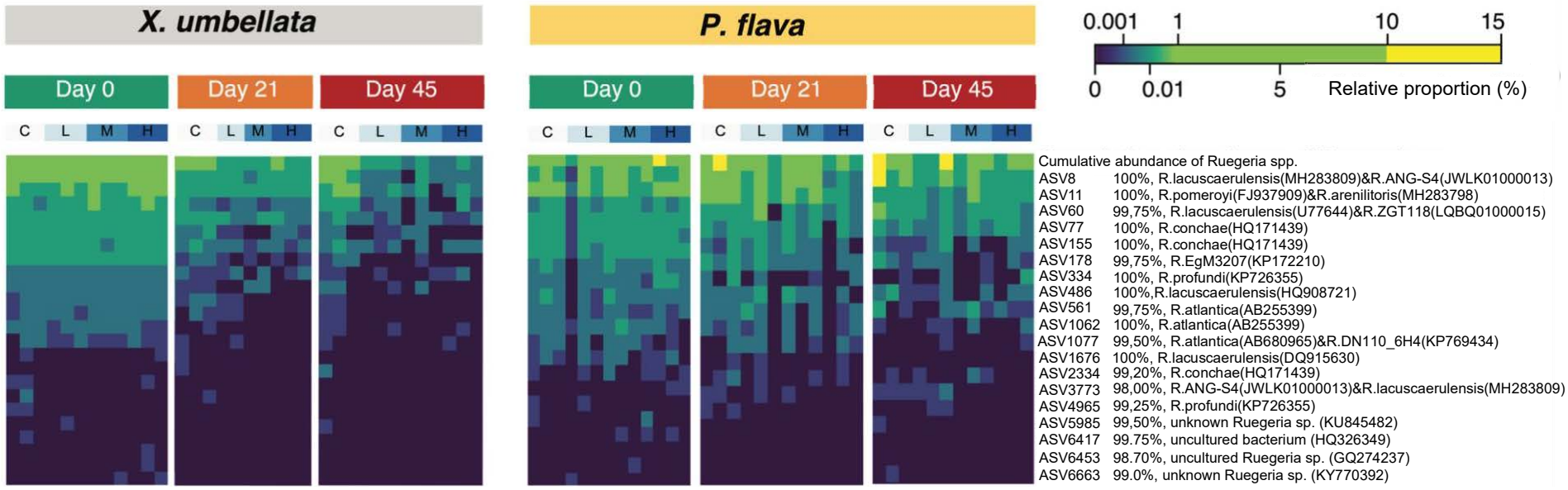

Fig. 3.4 Relative proportions of putative denitrifying *Ruegeria* spp. ASVs in total bacterial communities associated with corals *X. umbellata* and *P. flava* across different timepoints and DOC treatments.

The denitrifiers of *X. umbellata* revealed significant changes in community structure across treatments at day 45. This was primarily due to the reduced proportion of *Ruegeria* spp. under excess DOC and concomitant warming (**Fig. 3.3A; Fig. 3.4**). This shift in the community was further reflected in a significant decrease in *nirS* relative abundance in corals under excess DOC (**Fig. 3.3B**; ANOVA, $F_{6,24} = 2.45$, $P = 0.05$), especially when comparing the high DOC treatment (at a concentration of 40 mg L^{-1}) to the control (Tukey's HSD, $P = 0.03$). Conversely, denitrifiers in *P. flava* showed no significant change in community structure or cumulative relative abundance (**Fig. 3.3B;** *nirS* qPCR, ANOVA, $F_{6,24} = 0.93$, $P = 0.49$) in the presence of excess DOC and warming.

2.4.4 Physiological changes in *X. umbellata* subjected to excess DOC and warming

No change in phenotype was observed for the two octocoral species with excess DOC for 21 days. Even at the end of the experiment after combined excess DOC and warming conditions, *X. umbellata* still maintained a healthy appearance across excess DOC groups (**Fig. 3.5A**). In contrast, moderate bleaching was observed in *P. flava* under higher DOC concentrations (i.e., 20 mg L^{-1}, 40 mg L^{-1}). At day 45, *X. umbellata* with excess DOC showed 30% higher C:N ratios in host tissues compared to their control counterparts (**Fig. 3.5C;** ANOVA, $F_{3,8} = 12.59$, $P = 0.002$), while no significant difference was observed in C:N ratios of Symbiodiniaceae (ANOVA, $F_{3,8} = 1.002$, $P = 0.44$). This was accompanied by a moderate albeit not significant increase in host $\delta^{15}N$ signatures (**Fig. 3.5C;** ANOVA, $F_{3,8} = 2.7$, $P = 0.116$), as well as a 25% increase in $\delta^{15}N$ signatures of Symbiodiniaceae under excess DOC (ANOVA, $F_{3,8} = 19.44$, $P < 0.001$). There was no statistically significant difference across treatments in C:N ratios of *P. flava* host (**Fig. 3.5C;** ANOVA, $F_{3,8} = 2.435$, $P = 0.14$) and Symbiodiniaceae (ANOVA, $F_{3,8} = 2.508$, $P = 0.133$), nor in $\delta^{15}N$‰ signatures of host (ANOVA, $F_{3,8} = 1.396$, $P = 0.313$) and Symbiodiniaceae (ANOVA, $F_{3,8} = 0.234$, $P = 0.87$).

At the end of the experiment, seawater nutrient levels did not significantly differ among treatments with regard to NH_4^+ (**Fig. 3.5B;** ANOVA, $F_{3,8} = 0.069$, $P = 0.975$) and PO_4^{3-} (ANOVA: $F_{3,8} = 10.08$, $P = 0.969$) levels. Nevertheless, NO_2^- (0.22 ± 0.19 μM) and NO_3^- (1.60 ± 0.57 μM) concentrations from the control were two to four-fold higher (**Fig. 3.5B;** NO_2^-, ANOVA, $F_{3,8} = 1.502$, $P = 0.286$; NO_3^-, ANOVA, $F_{3,8} = 0.741$, $P = 0.557$) than those of aquaria with excess DOC (NO_2^-: 0.05 ± 0.04 μM; NO_3^-: 0.84 ± 0.66 μM).

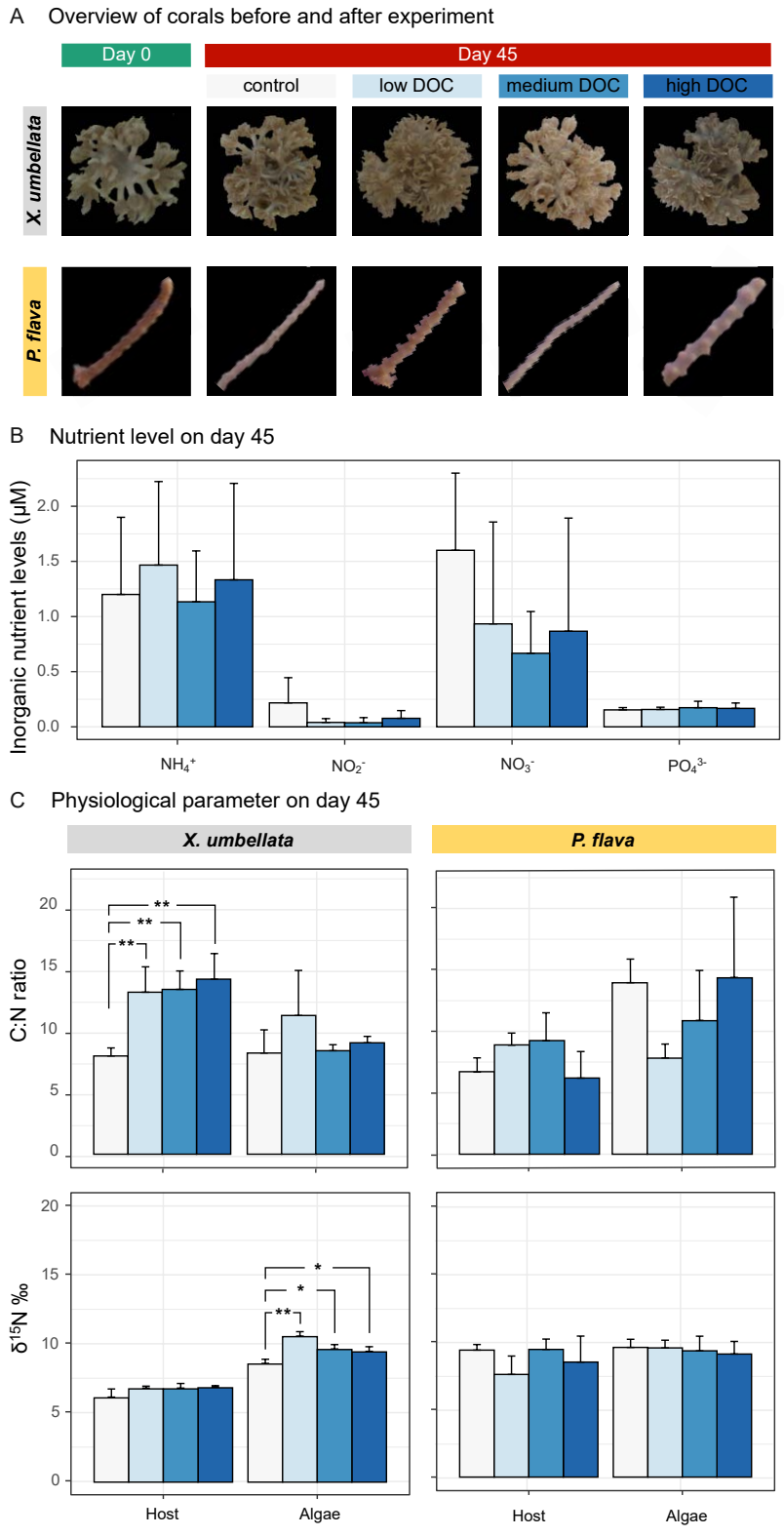

Fig. 3.5 Physiological changes corals *X. umbellata* and *P. flava* at the end of the experiment. **A** Phenotypes of corals *X. umbellata* and *P. flava* before and after the experiment. **B** Seawater inorganic nutrient levels in all aquaria at the end of the experiment. **C** Elemental (carbon and nitrogen) changes in both coral holobionts. Values are means ± SD, and the asterisk indicates statistically significant differences (*$p < 0.05$, **$p < 0.01$).

3.5 Discussion

N cycling microbes, including denitrifiers, are considered key players in the coral holobiont, as they might help in maintaining a N-limited state critical for the functioning of the coral-algal symbiosis (7, 49, 50). In the current study, we provide an assessment of the overall bacterial and denitrifying community structures associated with two octocoral species as well as their abundances and dynamics under excess DOC and concomitant warming in a six-week aquarium experiment. We found pronounced octocoral species-specific responses to excess DOC in bacterial community structure in general, and in denitrifier community composition and abundance, which aligned with patterns in C:N ratios of host tissues. Taken together, our results suggest a link between octocoral-associated denitrifiers and nutritional status, i.e. N availability of the holobiont, as well as a passive regulation of denitrifier communities as discussed below.

3.5.1 Distinct bacterial community structures between octocoral species

Bacterial community structure and diversity were distinct between two octocoral species *X. umbellata* and *P. flava* at the beginning of the experiment, even though these corals had been maintained in the same aquaria for years. At the bacterial class level, *P. flava* was initially dominated by *Alpha-* and *Gammaproteobacteria* as well as *Campylobacteria*; *X. umbellata* was dominated by *Alphaproteobebacteria*, *Bacteroidia*, and *Gammaproteobacteria*. Our results are in line with previous findings that octocoral-associated bacterial communities are species-specific (51-54). This also concurs with the abundantly reported host-specific microbial assemblages for scleractinian corals, as well as a structural similarity to scleractinian coral bacterial communities at the class level (15, 25, 30, 55, 56). The gorgonian *P. flava* showed a lower bacterial diversity, which is consistent with previous reports (52, 53).

Due to the broad leaf-like morphology and comparatively small polyps, algal symbionts may maximize photosynthetic activity to sustain a high autotrophic capacity in gorgonians (57). The invariable C:N ratio in the *P. flava* holobiont in our study suggests a relatively poor heterotrophic capacity, lending support to previous observations by Baker et al. (57). The low heterotrophic capacity of *P. flava* may restrict bacterial diversity (58). Limited niche space for bacterial associates due to morphological and physiological features of *P. flava* may be responsible for the observed low bacterial diversity. Being an octocoral, *P. flava* does not build a porous calcium carbonate skeleton that may offer niche space for a number of specialized

endolithic microbes, as known from scleractinian corals (59, 60), nor does it secrete a thick surface mucus layer like *X. umbellata* (61) that may provide favorable habitat for prokaryotic growth, including that of anaerobes (62, 63).

3.5.2 Species-specific responses of octocoral-associated bacterial communities to excess DOC

Little information on drivers of octocoral microbial dynamics is available. Here we showed that octocorals exhibit species-specific responses to excess DOC and concomitant warming with regard to their bacterial communities. Over time, excess DOC led to conspicuous changes in the bacterial community structure of *X. umbellata*, as characterized by a marked continuous increase in *Rhodobacteraceae,* making up to 80 % of the overall bacterial community at the end of the experiment. We cannot disentangle the direct and indirect effects of DOC on associated microbiomes. While based on qPCR data of 16S rRNA gene, we found an overall increase of bacterial abundance in *X. umbellata* under excess DOC. In contrast, no such change in response to excess DOC was observed in the bacterial community structure or abundance of *P. flava*. The absence of change in bacterial community structure in *P. flava* aligns with prior reports that gorgonian corals host a group of extremely robust bacterial communities over large geographic scales (64), seasonal variations (53) and even in the presence of environmental stressors such as increased temperatures and ultraviolet radiation (51). These observations suggest a differential 'flexibility' or plasticity in bacterial community responses, as recently reported for a number of scleractinian corals (65).

This apparent interspecific plasticity of octocoral microbiomes may be explained by the initial differences in bacterial diversity and community structure between these two octocoral species. In general, coral bacterial microbiomes with greater diversity likely show a higher degree of functional redundancy and may exhibit more flexible associations in response to environmental change, which align with the ecological resilience of the holobiont (10, 25, 66-68). In contrast, the lower diversity of the bacterial community associated with *P. flava* might suggest bacteria are highly selected and likely inhabit host-constructed niches, as previously reported (69). These may be dominated by a few specialized species of high abundance (65) and vary less under environmental perturbation (70).

Observations of such a species-specificity may pertain to functional traits of the animal host and/or algal symbionts, such as heterotrophic capacity, nutritional status, or ecological

resilience, including but not limited to heat tolerance as well as (a)biotic environmental drivers (25, 70-72). Differences in trophic strategies between these two octocoral species in particular may be of potential importance for their contrasting responses of the overall bacterial community under excess DOC. *Xenia* sp. is thought to be entirely autotrophic (73). However, Fabricius and colleague argued that *X. umbellata* may exhibit a mixotrophic lifestyle, i.e., relying on autotrophic and heterotrophic food sources (74). Our findings of increased C:N ratios in *X. umbellata* under excess DOC lend support to the latter. Indeed, the capacity to feed heterotrophically may allow *X. umbellata* to benefit from the surrounding DOC, thereby also creating a favorable environment for heterotrophic bacterial propagation, which may subsequently alter the overall bacterial community structure.

X. umbellata is known for its distinctive pulsation behavior, which may contribute to its ecological resilience by mixing of the surface boundary layer (75, 76). Of note, excess DOC remarkably enhanced their pulsation rates (77) and net primary productions (78), but whether this increase in pulsation may have an effect on the microbial plasticity of *X. umbellata* remains to be determined. Some endosymbiotic Symbiodiniaceae are known to feed heterotrophically (79), suggesting that excess DOC in *X. umbellata* may also promote the heterotrophic growth of Symbiodiniaceae. Given Symbiodiniaceae-associated bacteria often form a major component of the coral microbiome (80, 81), changes in Symbiodiniaceae physiology or even community composition likely contribute to microbial community dynamics observed in the present study.

Distinct patterns of microbial plasticity between two octocoral species appeared to be primarily driven by the increase of *Rhodobacteraceae* (ASV1: *Roseobacter* clade CHAB-I-5; ASV9 and ASV13: unclassified *Rhodobacteraceae*) exclusively in *X. umbellata*. This observation contrasts the findings of (65) reporting on the structural inflexibility in the bacterial microbiome of the scleractinian coral *Pocillopora verrucosa* under excess DOC. The pronounced increase in sequences proportions affiliated to multiple *Rhodobacteraceae* ASVs however align well with genomic evidence and ecological observations (82, 83). *Rhodobacteraceae* are known for their considerable versatility with regard to C utilization, which allows them to thrive and rapidly proliferate in high-DOC environments (82, 83). The 16S rRNA gene sequencing using hypervariable region V3-V4 could overestimate the prokaryotic diversity (93), however this overestimated diversity might cause negligible effects on our data. As we compared samples across the same species under different conditions, the

relative error could remain the same, hence the differences are still reflected by treatments. Additionally, the current study mainly focused on comparing dynamics of denitrifying bacterial abundance and community structure instead of surveying the total bacterial diversity between two octocoral species.

3.5.3 A passive regulation of denitrifiers in octocoral holobiont functioning

Coral-associated diazotrophs (N_2-fixing prokaryotes) have previously been suggested to play a central role in supporting holobiont fitness and functioning when the surrounding environmental N availability is low (7, 26). In contrast, denitrifiers have been proposed to be important in maintaining the coral-algal symbiosis in a N-limited state (7), yet our understanding of whether their abundance in the coral holobiont is of physiological or ecological relevance remains poor. On average, the proportion of putative denitrifiers in the microbiome was higher in *P. flava* than in *X. umbellata*. This difference between host species may be attributed to their distinct trophic strategies. Microbial community compositions in coral holobionts are highly selective due to different host functional or life history traits pertaining to e.g., development, physiology, and metabolism (17, 56, 84). Due to a relatively low capacity to obtain (in)organic N sources from the surrounding environment, *P. flava* is likely to rely more on symbiotic N cycling microbes to acquire or remove N to fulfill its metabolic requirements (13, 28, 85).

In the presence of N, bioavailable C sources including glucose usually favor denitrification and hence the growth of denitrifiers (86). However, excess glucose caused no stimulating effects on the relative abundance of denitrifiers in our study. This apparent contradiction might be explained by the following two considerations: first, denitrifier populations might be regulated by N availability; consequently, denitrifier abundance may not necessarily increase in the presence of high DOC loads in a N-limited environment. Second, if denitrifiers were not limited by environmental N availability, different denitrifying taxa may exhibit differential preferences for C sources. For instance, some *Rhodobacteraceae* taxa dominate microbial glucose uptake in coastal North Sea waters (87), while others may be suppressed by allochthonous glucose input, but follow fluctuations in population dynamics of primary producers (88).

Importantly, a reduction in the cumulative relative abundance of denitrifiers (as reflected in *nirS* relative gene copies quantified by qPCR) during DOC enrichment was exclusively observed in *X. umbellata*. In contrast to *P. flava*, this soft coral species showed an increase in C:N ratios in the animal host under excess DOC at the end of the experiment. As increased C:N ratios imply a relative decrease of N availability, associated changes in the nutritional status of the host may directly impact its interaction with associated denitrifiers. The notion of reduced N availability for holobiont members is further corroborated by the increase in $\delta^{15}N$ signatures, potentially indicating a reduced uptake of inorganic N from the seawater and an increased retention of N within the holobiont.

A passive regulation of N cyclers could thereby directly support overall holobiont functioning under fluctuating environmental conditions. In periods of low N availability, reduced denitrifier abundance (and therefore overall denitrification activity) might reduce the competition for N source in the coral holobiont and favor N uptake by the algae to support their growth. Likewise, rapid growth of denitrifiers during periods of excess N availability could increase the competition for N source between holobiont members, thereby alleviating excess N stress, and ultimately stabilizing the coral-algal symbiosis.

3.5.4 Can denitrifiers provide new insights into Symbiodiniaceae-bacteria interactions?

By inference, all four dominant denitrifiers identified in our study appear to form close associations with Symbiodiniaceae instead of coral hosts. The predominant putative denitrifier *Ruegeria* spp., formerly named *Silicibacter* spp., are known as dinoflagellate-associated bacteria. They are attracted to and capable of catabolizing degrading DMSP produced by the dinoflagellate host (89). *Labrenzia* spp. were previously reported to be 'core' microbiome members of Symbiodiniaceae in the coral holobiont (80, 81). Given their ability to produce DMSP (an osmolyte and powerful scavenger of reactive oxygen species), the consistent association of *Labrenzia* spp. potentially assist in reducing oxidative stress of Symbiodiniaceae (81). Likewise, *Dinoroseobacter* spp. were previously shown to supply their dinoflagellate host with essential nutrients, specifically the essential vitamins B_1 and B_{12}, to support their growth in particular under the nutrient-limitation state (90). *Ralstonia* spp. were identified as intracellular, i.e. occurring within the endodermal coral host cells in close proximity to the Symbiodiniaceae, and were proposed to be implicated in the functioning of the coral-algal symbiosis (69).

Notably, we observed differential responses of different putative denitrifying taxa to experimental treatments. Specifically, an increasing proportion of *Dinoroseobacter* spp. concurs with a decreasing proportion of *Ruegeria* spp. in *X. umbellata* with excess DOC. At this point, the causes and consequences of altered denitrifier community structure and abundance for octocoral holobiont functioning remain to be determined. Functional studies will be required to link these microbiome dynamics with related denitrifying activities and nutritional states of holobionts to disentangle the potential role of denitrifiers in octocoral holobiont fitness and functioning. Further localization of denitrifiers in the intact symbiosis could help us better understand the interactions between octocoral-associated N cycling microbes with other members of the holobiont: the animal host, Symbiodiniaceae, and other microbes in a changing environment.

3.6 Conclusion

Phase shifts on coral reef ecosystems have been linked to several environmental stressors. Among those, the detrimental effects of excess DOC (typically associated with sewage and reduced water quality) on scleractinian corals have received considerable attentions in the past (21, 22, 91). Changes in the activity, abundance and community structure of N cycling microbes have been discussed as critical components to corals' response to environmental DOC loading. While an imbalance in N cycling significantly affected the structure and functions of bacterial microbiomes associated with scleractinian corals, very little information is available for octocorals, which are globally abundant particularly on reefs undergoing phase shifts (92).

Here, we provide insights into the dynamics of octocoral-associated bacterial communities with an emphasis on putative denitrifying communities under excess DOC and warming. The dynamics of denitrifiers aligned with the nutritional status of the octocoral host, which implies their critical role in regulating internal nutrient availability of the holobiont in a changing environment. In order to obtain a better understanding on the interactions between octocoral holobiont members, future studies should expand to a comparative taxonomic framework of octocoral host species and link their functional and life history traits to their microbial communities, in particular potentially critical functional groups such as N cyclers. Integrated holistic approaches combining '-omics' approaches and cultivation-dependent methods may aid such a challenging endeavor.

3.7 Data Accessibility

The Sanger sequencing data derived from COI, mutS, and 28S rRNA gene PCR amplicons for octocoral identification have been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB43824. Primer-clipped DNA sequences generated by Miseq 16S rRNA amplicon sequencing were deposited on NCBI under BioProject accession number PRJNA718022.

3.8 Author Contributions

CW, AG, and NX designed and conceived the research; NX and SS performed aquarium project; CW and AG contributed coral samples and reagents; NX and CH analyzed sequencing data and conducted statistical analysis; NX conducted data visualization with improvement suggestions from CP and NR. NX wrote the paper with improvement suggestions from all of co-authors. All authors gave final approval for publication.

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3.10 Supplementary materials

3.10.1 Supplementary Figures

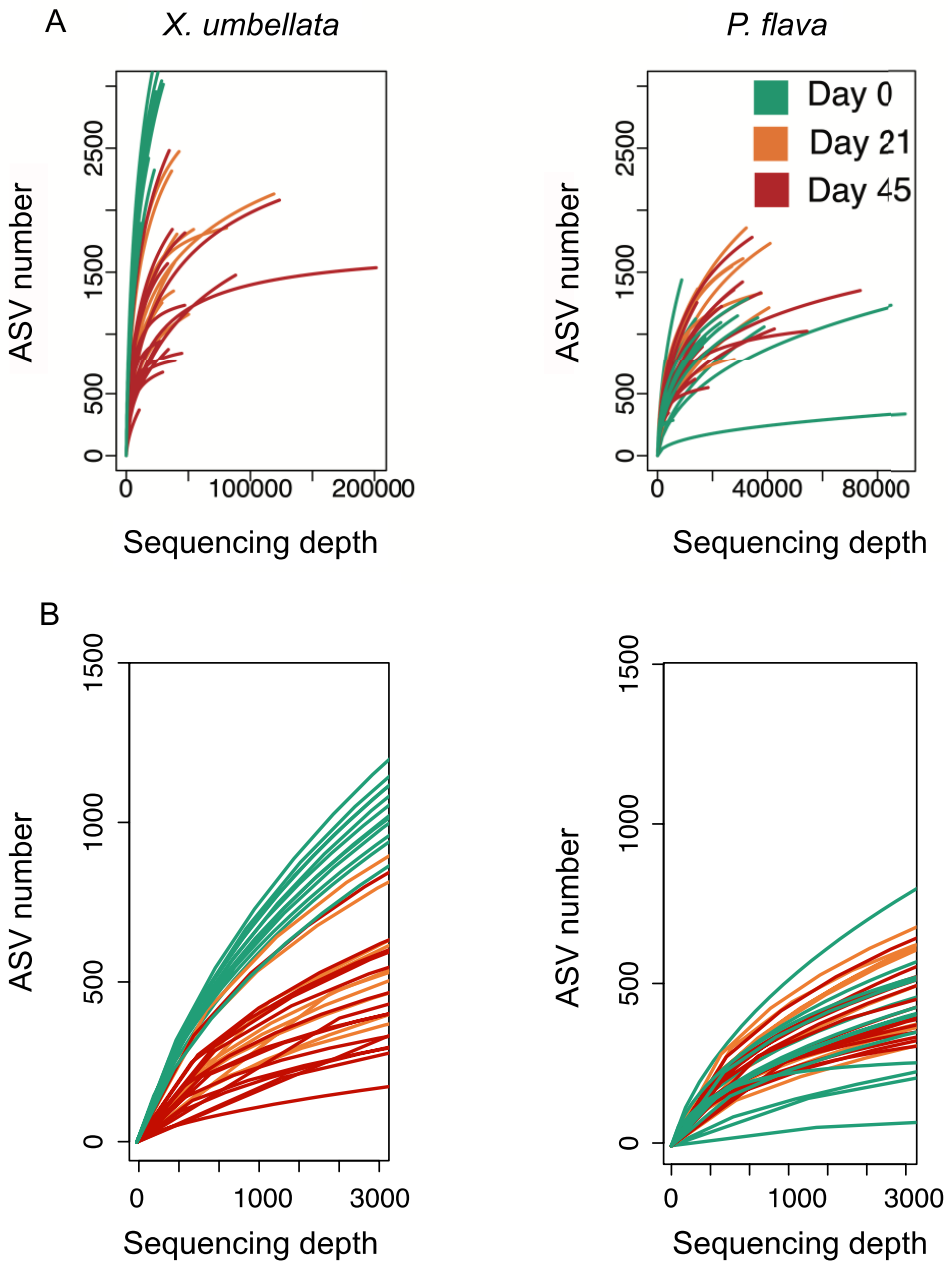

Fig. S3.1 Rarefaction curves of bacterial communities in corals *X. umbellata* and *P. flava*. **A** ASV numbers in *X. umbellata* with sequencing depth of 200,000 and in *P. flava* with sequencing depth of 80,000. **B** ASV numbers in corals *X. umbellata* and *P. flava* with sequencing depth of 3,000.

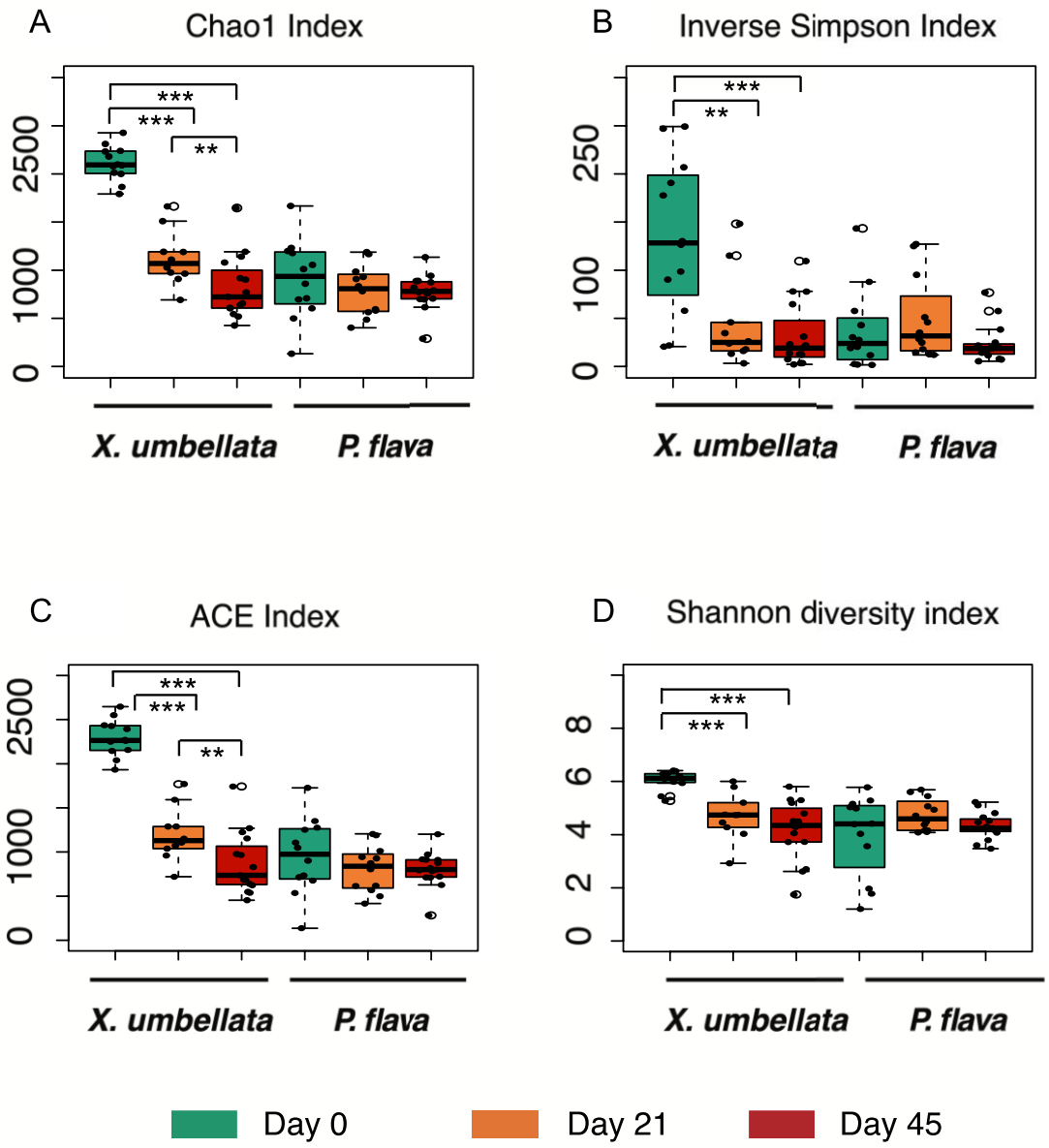

Fig. S3.2 Alpha diversity of bacterial communities in corals *X. umbellata* and *P. flava*. The asterisks indicate statistically significant differences compared to day 0 for each species separately (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **A** Chao1 index. **B** Inverse Simpson Index. **C** ACE index. **D** Shannon diversity index.

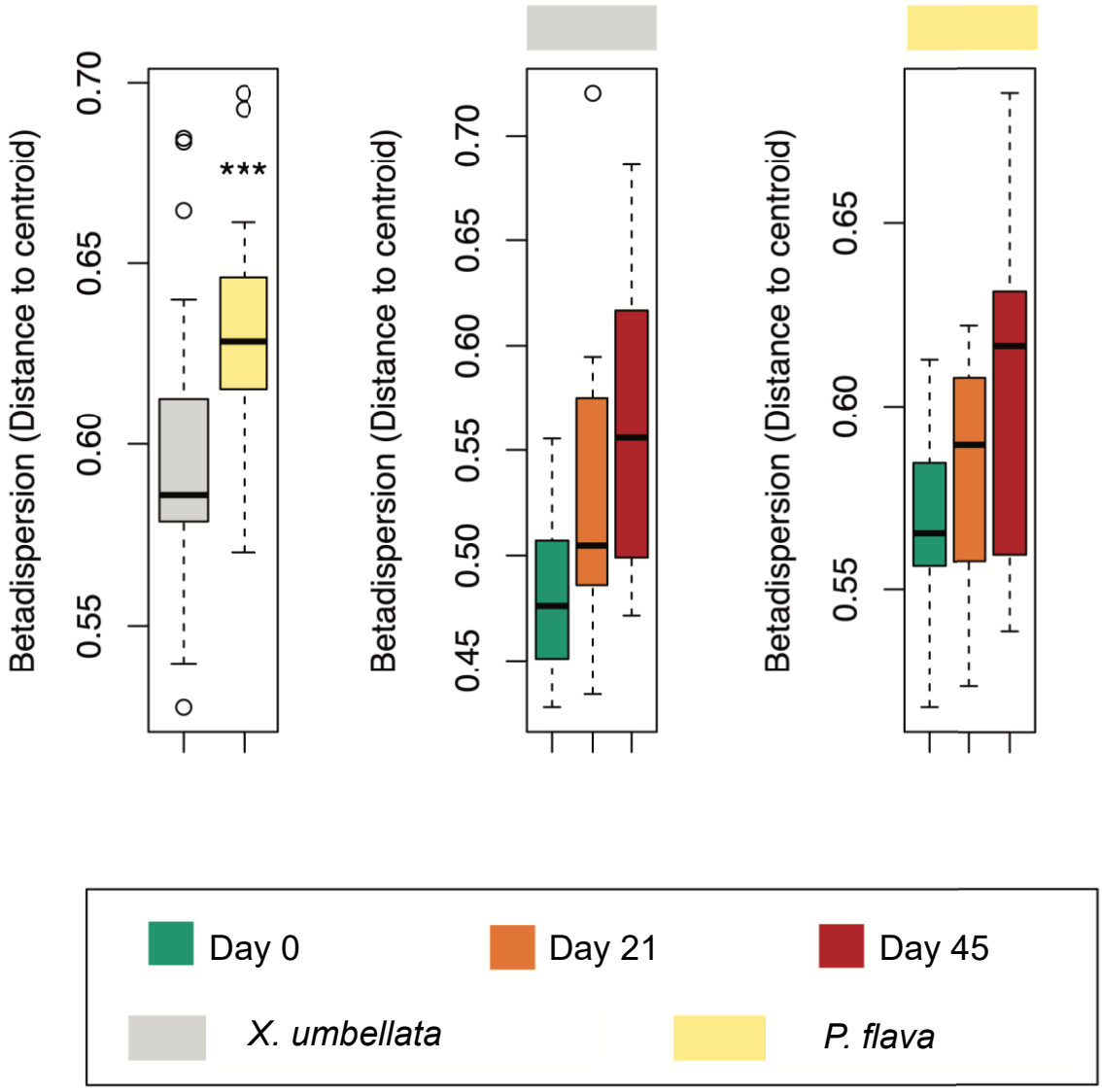

Fig. S3.3 Multivariate dispersion of bacterial communities in corals *X. umbellata* and *P. flava*. The asterisks indicate statistically significant differences between groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

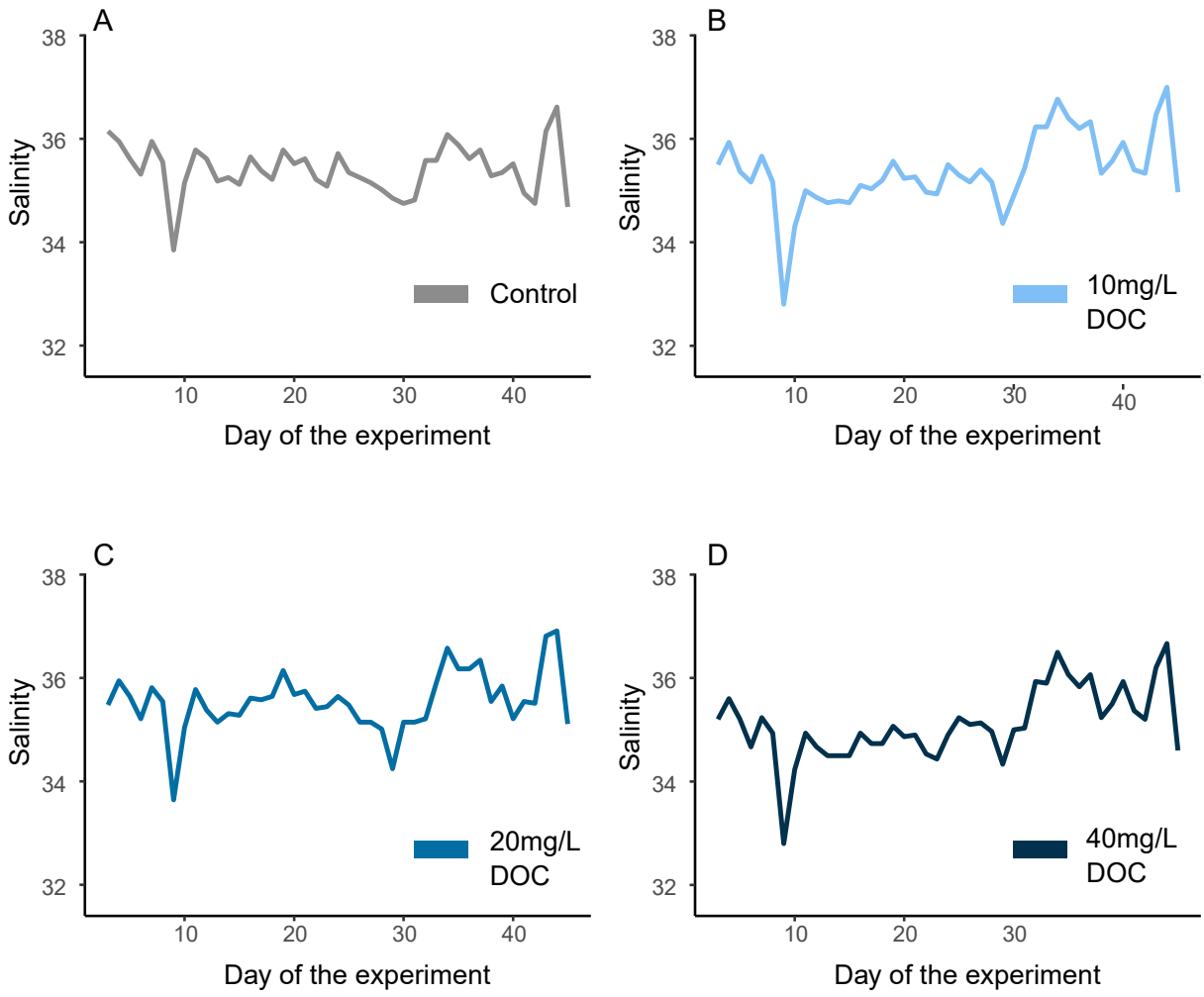

Fig. S3.4 The fluctuations in salinity (daily measurement) throughout the experiment. Values are shown by mean values for different treatments: **A** control; **B** low DOC (10 mg L^{-1}); **C** moderate DOC (20 mg L^{-1}); **D** high DOC (40 mg L^{-1}).

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Chapter 4 Presence of algal symbionts affects denitrifying bacterial communities in the coral model Aiptasia

Presence of algal symbionts affects denitrifying bacterial communities in the coral model Aiptasia

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Key words: Coral reefs, coral-algal symbiosis, nutrient cycling, denitrification, Symbiodiniaceae-bacteria interactions

Running Head: Denitrifying Prokaryotes in Cnidarian Symbiosis

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[++]These authors contributed equally to this work.

4.1 Abstract

The coral-algal symbiosis is maintained by a constant and limited nitrogen availability in the holobiont. Denitrifiers, i.e., prokaryotes reducing nitrate/nitrite to dinitrogen, could contribute to maintaining the nitrogen limitation in the coral holobiont, however the effect of host and algal identity on their community is still unknown. Using the coral model Aiptasia, we quantified and characterized the denitrifier community, in a full-factorial design combining two host and two strains of algal symbionts of the family Symbiodiniaceae. Strikingly, relative abundance of denitrifiers increased by up to 22-fold in photosymbiotic Aiptasia compared to their aposymbiotic (i.e., algal-depleted) counterparts. In line with this, while the denitrifier community in aposymbiotic Aiptasia was largely dominated by diet-associated *Halomonas*, we observed an increasing relative abundance of an unclassified bacterium in photosymbiotic CC7, and *Ketobacter* in photosymbiotic H2, respectively. Pronounced changes in denitrifier communities of Aiptasia with *Symbiodinium linucheae* strain SSA01 aligned with the higher photosynthetic carbon availability of these holobionts compared to Aiptasia with *Breviolum minutum* strain SSB01. Our results reveal that presence of algal symbionts increases abundance and alters community structure of denitrifiers in Aiptasia. Thereby, patterns in denitrifier community likely reflect the nutritional status of aposymbiotic vs. symbiotic holobionts. Such a passive regulation of denitrifiers may contribute to maintaining the nitrogen limitation required for the functioning of the cnidarian-algal symbiosis.

4.2 Introduction

Tropical coral reefs thrive in oligotrophic waters and are among the most diverse and productive ecosystems on Earth (1). The efficient nutrient recycling in the coral holobiont, i.e., assemblages of coral host, intracellular algal symbionts of the family Symbiodiniaceae, and a diverse community of prokaryotic microorganisms, is the key to this ecological success (2-5). In this photosymbiotic conglomerate, Symbiodiniaceae provide photosynthetically-fixed carbon to other holobiont members in exchange for carbon dioxide and inorganic nutrients from the coral host (6). Importantly, the efficient carbon recycling in the holobiont is maintained by limited nitrogen availability for the algae (7). A disruption of nitrogen limitation in the holobiont may promote uncontrolled growth and retention of photosynthates by algal symbionts (8, 9). Thereby, the functioning of coral holobionts under anthropogenic change depends in part on their ability to maintain a nitrogen-limited state for the algal symbionts (10).

The taxonomically and functionally diverse prokaryotic microbiome associated with corals (11-15) contributes to holobiont nutrient cycling (16-19). Diazotrophs, i.e., prokaryotes fixing atmospheric dinitrogen into ammonia, are ubiquitous members of coral holobionts (20-25). Diazotrophs have been widely considered beneficial for corals on pristine oligotrophic reefs, as they supply 'new' nitrogen to help sustain coral productivity during low environmental nitrogen availability (19). Conversely, stimulated diazotroph activity due to ocean warming and organic carbon eutrophication has been linked to the destabilization of the coral-algal symbiosis by disrupting nitrogen limitation in the holobiont (9, 21, 26). In contrast, denitrifiers, i.e., prokaryotes that reduce nitrate/nitrite to dinitrogen (27), have been barely explored in the coral holobiont. However, denitrifiers could be key players in coral holobiont fitness and functioning, as they have the potential to help in maintaining nitrogen-limited conditions (10, 28-30).

Denitrifiers are ubiquitously associated with benthic reef organisms and likely show host-specific community compositions (28, 30). A positive correlation of denitrifying activities in the coral holobiont with elevated nutrient concentrations in reef waters has been reported (31), which lends tentative support to the notion that denitrifiers may help corals survive eutrophic conditions by maintaining nitrogen limitation within the holobiont (28-30). Further, as denitrifying activity and Symbiodiniaceae density exhibit positive correlations in hard corals (28), it has been suggested that denitrifiers and Symbiodiniaceae may intimately interact within the coral holobiont, and that the former are limited by photosynthates released by the latter (28). Indeed, denitrification is an energy-consuming process, and the denitrifying activity is typically

limited by organic carbon availability (32). Finally, a recent effort combining 16S rRNA gene sequencing with functional inference suggests that a majority of denitrifiers in octocorals are members of the Symbiodiniaceae core microbiome (30). Still, the factors driving denitrifier communities in the coral holobiont remain largely speculative; the effect of host and algal identity on the structuring of denitrifier communities remains unexplored.

Disentangling the respective contribution of host and algal symbionts to the structuring of prokaryotic communities is challenging in the intact coral holobiont, as it is difficult to maintain corals in aposymbiotic (i.e., algal-depleted) state and to manipulate their algal communities (33, 34). In contrast, owing to the unique advantages of being amenable to manipulations of algal symbiont communities, the sea anemone *Exaiptasia diaphana* (from here on referred to as "Aiptasia") is a model organism commonly used for functional studies on metabolic interactions in the coral-algal symbiosis, (35-37). Here, we set out to assess the contribution of host and algal symbiont identity as drivers of denitrifier abundance and community composition in a full-factorial design, combining two Aiptasia strains in aposymbiotic state or symbiotic with two different Symbiodiniaceae clonal lineages (**Fig. S4.1**). In addition, we assess the physiology and nutritional status of holobionts by measurements of symbiont density, photosynthetic efficiency, and carbon to nitrogen ratio. Thereby, we addressed the following questions: 1) Do denitrifier communities in Aiptasia differ between photosymbiotic states? 2) If so, does algal or host identity matter? And, finally: 3) Do patterns in denitrifier abundance and community composition reflect the nutritional status of holobionts?

4.3 Materials and Methods

4.3.1 Aiptasia rearing, experimental design, and sampling

Anemones of the clonal anemone Exaiptasia diaphana ('Aiptasia') strains CC7 (North Carolina) (38) and H2 (Hawaii) (39) were kept in separate 3 L containers filled with artificial seawater (salinity 35 PSU; Pro Reef, Tropic Marine, Germany) at 25 °C with a 12 h:12 h light/dark cycle at 60 µE m^{-2} s^{-1}. Aiptasia were fed with freshly prepared brine shrimp (*Artemia salina*) nauplii once a week, followed by tank cleaning and full water exchange the next day. The two Aiptasia strains CC7 and H2 are genetically distinct and differ in their native dominant endosymbiont (40). Aiptasia CC7 is natively associated with the symbiont SSA01, a strain of *Symbiodinium linucheae* (38, 41). The Aiptasia H2 is natively associated with the symbiont SSB01, a strain of *Breviolum minutum* (39, 42).

Briefly, photosymbiotic Aiptasia were depleted of their native symbiont by incubation in 4 ºC seawater for 4 h, followed by 2 days at 25 ºC in artificial seawater containing the photosynthesis inhibitor diuron as outlined previously (Pringle lab 2018. Cold-shock protocol to bleach Aiptasia. protocols.io https://dx.doi.org/10.17504/protocols.io.qx8dxrw). The aposymbiotic Aiptasia were maintained in the dark for more than six months. Prior to experiments, the absence of symbiont was confirmed via a lack of chlorophyll autofluorescence, assessed by fluorescence stereomicroscopy. For inoculations of Aiptasia with algal symbionts, the aposymbiotic animals were incubated twice in artificial seawater containing 10^5 Symbiodiniaceae cells mL^{-1} during the feeding with *Artemia salina* nauplii in two consecutive weeks. Thereby, after two months, our experimental design generated six distinct host-symbiont combinations of Aiptasia: aposymbiotic CC7 (CC7-APO), CC7 with symbiont SSA01 (CC7-SSA01), CC7 with symbiont SSB01 (CC7-SSB01); aposymbiotic H2 (H2-APO), H2 with symbiont SSA01 (H2-SSA01), H2 with symbiont SSB01 (H2-SSB01) (for a detailed overview of the experimental setup, refer to **Fig. S1**). Six host-symbiont combinations were maintained in different tanks containing 10 animals each. This allowed us to disentangle the contribution of host and algal identity to the structuring of denitrifier communities in three comparisons: a) between aposymbiotic and photosymbiotic states of the same host, b) between different symbionts within the same host, and c) between different hosts with the same symbiont.

4.3.2 Assessment of host tissue protein content and Symbiodiniaceae cell density

For Aiptasia sampling, five replicates were collected for each group, in total 5 replicates * 6 groups = 30 samples. Each Aiptasia was homogenized with 500 µL filtered (0.22 µm) 2X phosphate buffered saline (PBS) in a 1.5 mL sterile Eppendorf tube using a cordless Pellet Pestle™ (Fisherbrand, USA). For the measurement of host protein content, 100 µL homogenate aliquots were centrifuged at 3,000 × g for 3 min using Centrifuge 5910R (Eppendorf, Germany). For each sample, three technical replicates of 5 µL of the five-fold diluted supernatant were transferred into a 96-well plate. The protein content was quantified using the Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, US) following the manufacturer's protocol and recording the absorbance at 595 nm using a CLARIOstar Plus plate reader (BMG LABTECH, Germany). For Symbiodiniaceae density, 100 µL aliquots of homogenate were centrifuged at 3,000 × g for 3 min. 500 µL filtered 2X PBS was added to resuspend the Symbiodiniaceae pellets. The Symbiodiniaceae density was quantified for 6 technical replicate aliquots for each Aiptasia sample using a Countess II FL (Invitrogen, USA) fluorescence cell counter reading from channel CY5.

4.3.3 Photosynthetic efficiency and analysis of elemental carbon and nitrogen

The maximum photosynthetic efficiency of six host-symbiont combinations was assessed *via* pulse-amplitude modulation (PAM) fluorometry. Aiptasia were dark-adapted for 30 min and the ratio between variable and maximum chlorophyll fluorescence (F_v/F_m) was measured using the blue version of the MINI-PAM-II (Walz, Germany) with a 5.5-mm fiber optic. For measuring the elemental C and N contents, Aiptasia animals were collected and homogenized with 500 µL filtered 2X PBS in a 1.5 mL sterile Eppendorf tube. Homogenized tissues were transferred on a clean 0.22 µm filter and dried at 40 °C until constant weight. Around 5 mg of dried sample was used to measure total C and N content using a Euro EA-CHNSO Elemental Analyser (HEKAtech, Germany).

4.3.4 DNA extraction, nirS *amplification, and quantitative PCR (qPCR)*

Genomic DNA (including 3 negative extractions) were extracted with the DNeasy Blood and Tissue Kit according to manufacturer instructions (Qiagen, Germany). Extracted DNA was quality-checked by spectrophotometry at 260 nm and 280 nm on a Nanodrop 2000C spectrophotometer (Thermo Scientific, USA), and quantified using a Qubit and the dsDNA High Sensitivity Assay Kit (Invitrogen, USA).

The relative abundance of Symbiodiniaceae strains in symbiotic animals was quantified by qPCR with genus-specific primer pairs using a qTOWER3 84G (Analytik Jena, Germany) with the innuMIX qPCR DSGreen Standard kit (Analytik Jena, Germany). Each qPCR reaction consisted of: 5 µL of MasterMix, 0.5 µL of 10 µM forward and reverse primer each, 3 µL of nuclease-free water, and 1 µL DNA template (10 ng μL^{-1}) for a total reaction volume of 10 µL. The thermal cycling condition consisted of a hot-start activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Final extension was carried out at 72 °C for 10 s followed by a melting curve from 65 °C to 95 °C with an increase of 0.5 °C every 5 s. Samples were amplified using the *Symbiodinium*-specific primer pair A-F (5'-CCTCTTGGACCTTCCACAAC-3') and A-R (5'-GCATGCAGCAACACTGCTC-3') as well as the *Breviolum*-specific primer pair B-F (5'-GTCTTTGTGAGCCTTGAGC-3') and B-R (5'-GCACACTAACAAGTGTACCATG-3') (43). To obtain the relative proportions of these two Symbiodiniaceae genera (clades) in each sample, the ΔCt for each sample was calculated based on the cycle threshold (Ct) values of

Symbiodiniaceae SSA01 against Ct values of SSB01, followed by a reference against the ΔCt of DNA extracted from a 1:1 ratio of SSA01 and SSB01 cells using the ΔΔCt method (44). The qPCR efficiency for *Symbiodinium*-specific and *Breviolum*-specific amplifications were 95.28% and 97.45%, respectively.

The nitrite reductase *nirS* gene was used as a marker to quantify and characterize the denitrifier community (27). Following the same reagent conditions for qPCR reactions as outlined above, samples were amplified by the thermal cycling condition consisted of a hot-start activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C (*nirS*) for 30 s and extension at 72 °C for 30 s. Final extension was carried out at 72 °C for 10 s followed by a melting curve from 65 °C to 95 °C with an increase of 0.5 °C every 5 s. A fraction of the *nirS* gene was amplified using the primer pair nirS-1F (5'-CCTAYTGGCCGCCRCART-3') and nirS-qR (5'-TCCMAGCCRCCRTCRTGCAG-3') (45) and the specificity of amplification was confirmed with Sanger sequencing (Eurofins Genomics, Germany). Relative fold change of the *nirS* gene was referenced to the mean Ct of aposymbiotic CC7 by ΔCt method with normalized genomic DNA quantity (46). The qPCR efficiency for *nirS* gene amplification was 100.80%.

4.3.5 nirS *gene amplicon library preparation and sequencing*

To characterize the denitrifier community composition, the *nirS* primer pair 1F, qR (previously used for qPCR as outlined above) with sample-specific paired NovaSeq barcodes was used to amplify a region of the *nirS* gene. PCR was run using a reaction volume of 10 µL containing 5 µL of Qiagen multiplex PCR master mix (Qiagen, Germany), 1 µL of 5 µM forward and reverse primer each, 2 µL nuclease-free water, and 1 µL DNA template (10 ng μL^{-1}). Thermal cycling condition consisted of an initial activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 65 °C for 20 s, and 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min. The PCR products were quality-checked on a 1 % gel via electrophoresis using EPS 150/2000 (VWR, USA) and purified using one-Step ExoProStar (Fisher Scientific, USA). Purified PCR products were indexed using the Nextera XT Index Kit v2 (Illumina, USA) according to manufacturer instructions. Indexed samples were cleaned and normalized using the SequalPrep Normalization Plate Kit (Invitrogen, USA). All samples were pooled in one 1.5 mL Eppendorf tube and concentrated to 100 µl using a Vacuum Concentrator plus (Eppendorf, Germany). The final DNA yield of pooled 48 samples (distributed over 34

Aiptasia animals in 6 distinct host-symbiont combinations, 3 SSA01 algal culture aliquots, 3 SSB01 algal culture aliquots, 3 Artemia culture aliquots, 3 negative control DNA extractions, 2 negative control PCR reactions) was 414 ng determined by Qubit dsDNA High Sensitivity Assay (Invitrogen, USA). Sequencing was conducted by Novogene (Cambridge, UK) on the NovaSeq 6000 PE250 platform (Illumina, USA) using 2*250 bp paired-end reads.

4.3.6 Sequence processing and analysis

For *nirS* amplicons, sequence quality control was conducted in R (version 4.1.0) following the DADA2 Pipeline Workflow (version 1.22.0) with slight modifications (47). Primers were removed from the demultiplexed sequences with "Cutadapt" v.2.10 (48). Due to the biological length variation of *nirS* gene (27), the paired-end reads were quality filtered by the minimum length of 100 bp, as determined by the length distribution and identity check. Reads were de-replicated and error rates were estimated and used for inference of true amplicon sequence variants (ASV). After merging paired reads, a *nirS* ASV table was constructed with chimera removal by the *de novo* approach. ASVs were filtered by hits of gene "*nirS*" after Blastx against the UniprotKB/Swiss-Prot database (49).

Remaining ASVs were filtered by the length of 220 - 240 bp, as determined by the length distribution, and were further translated to the protein level in a correct open reading frame using TranslatorX (50) and ORFfinder in NCBI. The *nirS* protein sequences were aligned by multiple alignment in MUSCLE 3.8.425 (51). A *nirS* phylogenetic tree was built using Geneious Tree Builder in Geneious Prime (version 2021.0.3) (52). For taxonomic assignment, a protein sequence database of the *nirS* gene was downloaded from the FunGene repository (53) (accessed 10/01/2022). Sequences belonging to uncultured organisms were excluded, which resulted in a customized FunGene database containing 3,504 *nirS* gene sequences with known taxonomy across 238 genera and 894 species. Query *nirS* protein sequences were run with blastp against the customized FunGene database for the taxonomic assignment. Determined *nirS* sequencing data are available on NCBI under BioProject PRJNA836569. All bioinformatic workflows can be accessed at: https://github.com/NancyXiang/nirS-Sequence-Analysis.

4.3.7 Statistical analysis

Data analysis and plotting were conducted in R (version 4.1.0) using several packages, such as "ggplot2" (54) and "vegan" (55). After log transformation to meet data normality, symbiont density, C:N ratio, and photosynthetic efficiency were analyzed by two-way analysis of variance (ANOVA) defining host strains (2 levels: CC7 and H2) and photosymbiotic associations (3 levels: aposymbiotic, SSA01 photosymbiotic and SSB01 photosymbiotic) as two factors followed by Tukey's honestly significant difference (HSD) as a post hoc comparison. For non-normally distributed qPCR data, Kruskal–Wallis were used with Dunn test as post hoc comparisons.

For *nirS* sequencing data, ASVs were considered putative contaminants if their relative abundances were >10% in negative controls. Alpha diversity was calculated based on repeated random subsampling of ASVs to the minimum library size of 12,521 reads. Statistical differences were tested for each Alpha diversity index using a two-way ANOVA with Tukey's HSD. Beta diversity was calculated using Euclidean distances of centered log-ratio (clr)-transformed ASV counts. Statistical differences in beta diversity between host strains and across photosymbiotic associations were tested using Permutational multivariate analysis of variance (PERMANOVA, 'adonis' function, 999 permutations) and represented on a Principal component analysis (PCA) ordination using Phyloseq v1.38.0 (56). Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (57) was used to identify differentially abundant ASVs between host strains and between photosymbiotic associations within each host.

4.4 Results

4.4.1 Algal depletion and inoculation in Aiptasia

Inoculations of two aposymbiotic Aiptasia strains with two different algal strains were confirmed using fluorescence microscopy (**Fig. 4.1A**) and counts of algal density in host tissues (**Fig. 4.1B**). The algal density significantly differed between host strains (**Fig. 4.1B**; two-way ANOVA, $F_{1,16}$, $P = 0.001$) and across photosymbiotic associations (**Fig. 4.1B**; two-way ANOVA, $F_{2,16}$, $P < 0.001$). While aposymbiotic CC7 showed no detectable algae by fluorescence cell counter, genus-specific qPCR identified a background population of algal symbionts dominated by *Symbiodinium linucheae* strain SSA01, making up 97.3% of the total Symbiodiniaceae community. Likewise, a low number of algal cells mg^{-1} host protein were detected in aposymbiotic H2, with *Breviolum minutum* strain SSB01 dominating 80.1% of the total Symbiodiniaceae community (**Fig. 4.1B**). Aiptasia with algal symbionts had significantly

higher algal densities than their aposymbiotic counterparts (**Fig. 4.1B**; Tukey HSD of CC7, $P < 0.001$; Tukey HSD of H2, $P < 0.001$). Aiptasia CC7 and H2 accommodated non-significantly higher algal densities per mg host protein when associated with symbiont SSA01, compared to when associated with symbiont SSB01 (**Fig. 4.1B**; Tukey HSD, $P = 0.856$ and $P = 0.998$, respectively). In Aiptasia with algal symbionts, the respective algal strain used for inoculations dominated the Symbiodiniaceae community of CC7 and H2 (SSA01: > 99.99 and 85.32%, respectively; SSB01: 90.73 and 88.84 %, respectively).

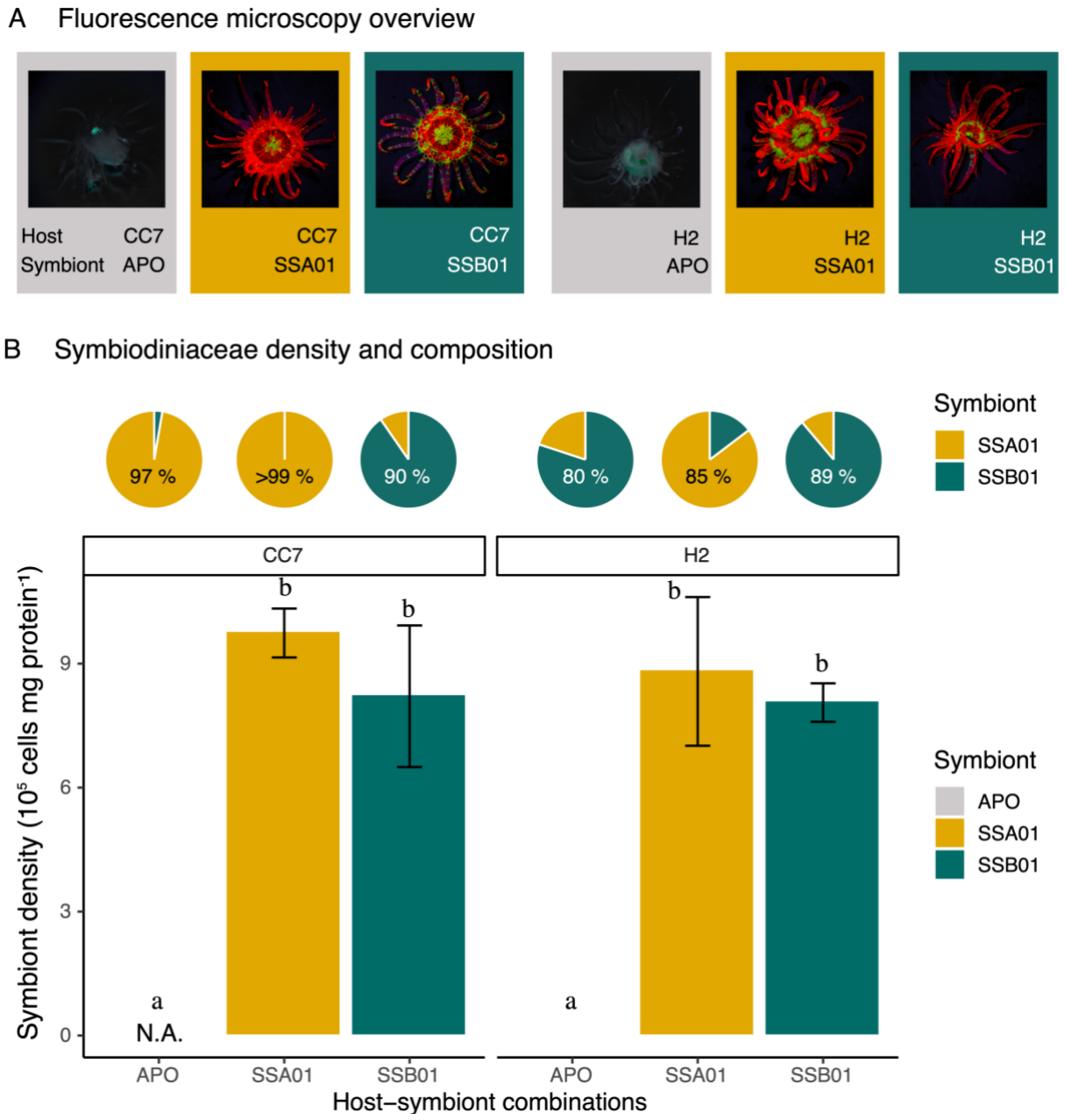

Fig. 4.1 Inoculations of aposymbiotic Aiptasia with algal symbionts. A Fluorescence microscopy overview of six host-symbiont combinations to visualize *in hospite* chlorophyll of photosymbiotic Symbiodiniaceae. **B** Symbiodiniaceae density and community composition of six host-symbiont combinations. All data are shown as mean ± SE (n = 5 animals each). Different letters above bars indicate significant differences between groups (two-way ANOVA with Tukey HSD, $P < 0.05$).

4.4.2 High carbon availability in Aiptasia with symbiont SSA01 aligns with photo-physiological performance

Aiptasia with SSA01 showed significantly higher C:N ratios than their SSB01 photosymbiotic or aposymbiotic counterparts (**Fig. 4.2A**; two-way ANOVA, $F_{2,24}$, $P < 0.001$), regardless of the host identity (two-way ANOVA, $F_{1,24}$, $P = 0.585$). Aiptasia CC7 showed a fairly stable C:N ratio across photosymbiotic associations (TukeyHSD, P = 0.129-0.869). In contrast, Aiptasia H2 with symbiont SSA01 showed ~50% and ~70% higher C:N ratio compared to those with symbiont SSB01 (TukeyHSD, P = 0.024) and their aposymbiotic counterparts (TukeyHSD, P = 0.001), respectively. Higher photosynthetic efficiency was found in Aiptasia with symbiont SSA01 than those with SSB01 (**Fig. 4.2B**; two-way ANOVA, $F_{1,16}$, P = 0.004; TukeyHSD of CC7, P = 0.009; TukeyHSD of H2, P = 0.723).

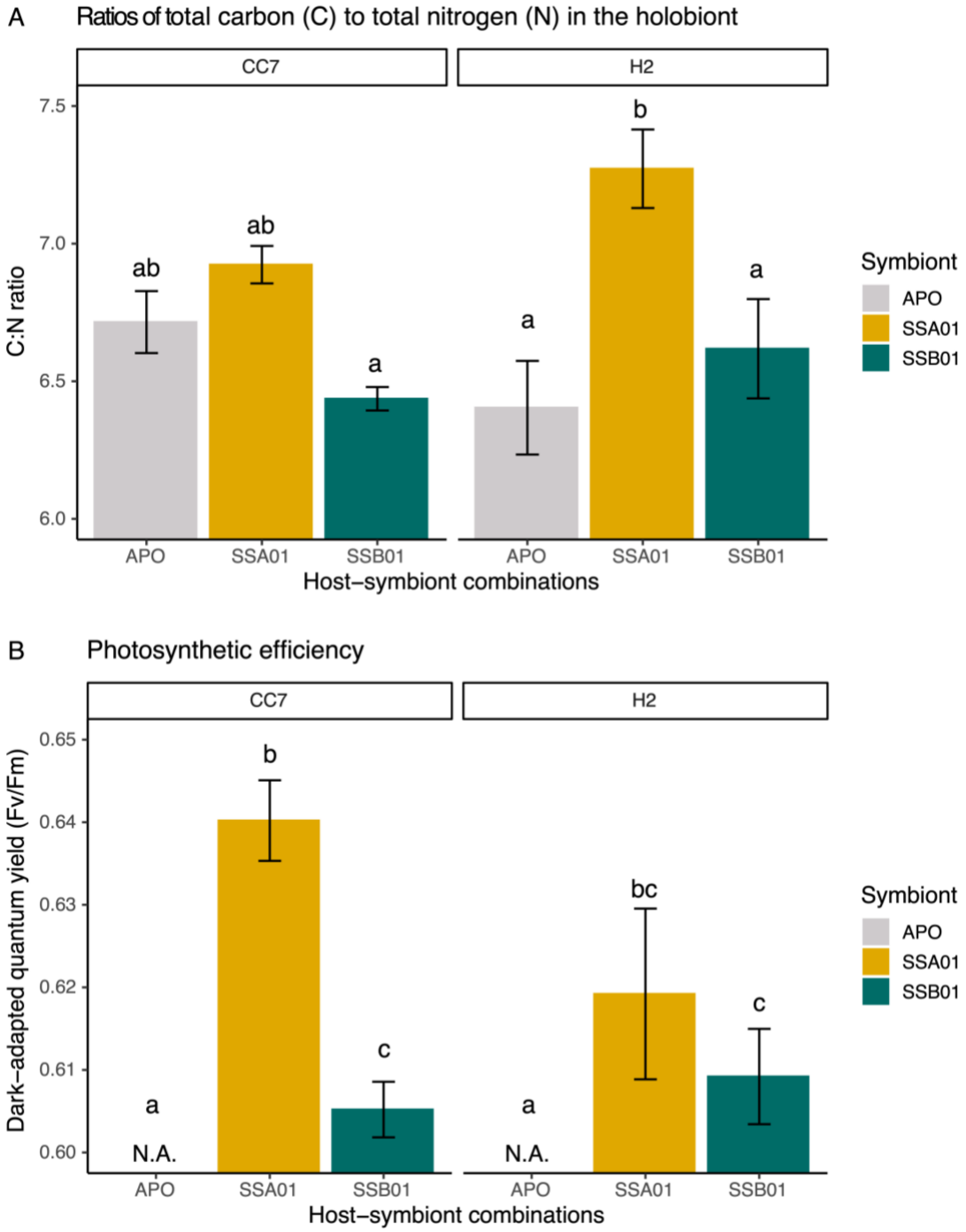

Fig. 4.2 Physiology of six host-symbiont combinations of Aiptasia. A Ratios of total carbon to total nitrogen. **B** Photosynthetic efficiency of photosystem II. All data are presented as mean ± SE (n = 5 animals each). Different letters above bars indicate significant differences between groups (two-way ANOVA with Tukey HSD, $P < 0.05$).

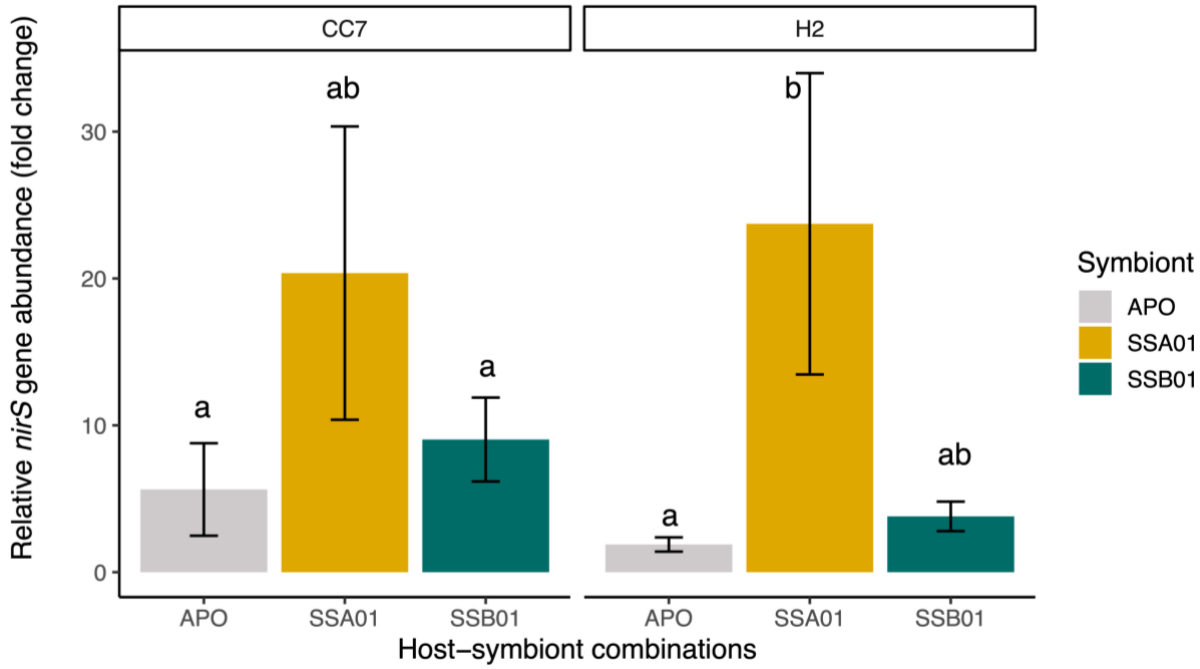

Fig. 4.3 Relative abundance of denitrifiers. Relative fold change in copy numbers of the *nirS* gene in relation to the mean Ct of aposymbiotic CC7 samples. DNA inputs for qPCR are normalized. All data are presented as mean ± SE (n = 5 animals each). Different letters above bars indicate significant differences between groups (Kruskal–Wallis with Dunn test, $P < 0.05$).

4.4.3 Higher denitrifier abundance in photosymbiotic Aiptasia than aposymbiotic counterparts

Relative *nirS* gene copy numbers, i.e., relative abundances of denitrifiers quantified by *nirS* qPCR did not differ between host strains (**Fig. 4.3**; Kruskal–Wallis, $P = 0.438$), but significantly differed across photosymbiotic associations (Kruskal–Wallis, $P = 0.006$). The relative abundance of denitrifiers in Aiptasia with symbiont SSA01 were nearly 4- to 23-fold higher compared to their SSB01 photosymbiotic (Kruskal–Wallis, $P = 0.135$) and aposymbiotic counterparts (Kruskal–Wallis, $P = 0.004$).

4.4.4 *nirS* sequencing overview

After demultiplexing samples and processing raw reads with the DADA2 pipeline, we obtained 1,104 ASVs represented by 7,414,102 reads. We retained 616 ASVs (represented by 6,907,743 reads) after confirming their functional annotation to *nirS* by Blastx against SwissProt, from which we kept 565 ASVs represented by 6,907,542 reads that had a length of 220-240 bp. Of these, 558 ASVs represented by 6,905,196 reads were successfully translated to *nirS* proteins

in a correct open reading frame. After discarding ASVs considered as contaminants, 427 ASVs represented by 5,378,208 reads distributed over 48 samples were retained for downstream statistical analysis and visualization. Of these, 305 ASVs (386,704,8 reads) were assigned to a taxonomic group as opposed to 122 ASVs which could not be classified.

A total of 27 genera of denitrifiers were identified across 43 samples (including 34 host-symbiont combinations of Aiptasia animals, 6 algal culture samples and 3 food samples). The sequencing data were highly dominated by a few abundant ASVs, three dominant taxa *Halomonas* (ASV1; Gammaproteobacteria, *Halomonadaceae*), an unclassified bacterial symbiont of *Nonionella stella* (ASV2) and *Ketobacter* (ASV3; Gammaproteobacteria, *Alcanivoracaceae*) made up to 83.06% of the total sequencing reads (33.44%, 26.64%, and 22.98%, respectively). The alpha diversity indices showed no significant difference between host strains (**Fig. S4.2**; Chao1 richness: $F_{1,26} = 0.004$, P=0.952; Shannon diversity: $F_{1,26} = 0.508$; P = 0.482; Simpson evenness: $F_{1,26} = 0.292$, P = 0.594) or across photosymbiotic associations (Chao1 richness: $F_{2,26} = 1.351$, P = 0.277; Shannon diversity: $F_{2,26} = 0.355$; P = 0.704; Simpson evenness: $F_{2,26} = 0.323$; P = 0.727).

4.4.5 Pronounced shift of denitrifier community composition aligns with algal photo-physiology

The denitrifier community compositions were distinct among Aiptasia animals, algal culture samples, and food samples (**Fig. 4.4A, Fig. 4.4B**; PERMANOVA, $F_{2,38} = 1.31$, P = 0.045). The predominant ASV1 (Gammaproteobacteria, *Halomonadaceae*, *Halomonas*) in food samples (~80% of the total sequences) showed its prevalence across the entire dataset (**Fig. 4.4B**). Focusing on Aiptasia, their denitrifier community composition was affected by both host identity (PERMANOVA, $F_{1,30} = 2.20$, P = 0.001) and photosymbiotic association (PERMANOVA, $F_{2,29} = 1.27$, P = 0.038).

Denitrifier communities in aposymbiotic CC7 and H2 showed no significant difference and were both highly dominated by ASV1 (**Fig. 4.4B**; PERMANOVA, $F_{1,10} = 1.00$, P = 0.473). After inoculations with algal symbionts, the denitrifier community compositions varied considerably in both Aiptasia strains (PERMANOVA of CC7: $F_{2,14} = 1.41$, P = 0.002; PERMANOVA of H2: $F_{2,12} = 1.34$, P = 0.024). The *post hoc* analysis identified significant differences between Aiptasia with symbiont SSA01 and their aposymbiotic counterparts (PERMANOVA pairwise of CC7: F = 1.83, P = 0.003; PERMANOVA pairwise of H2: F =

1.62, P = 0.048). The effects of symbiont SSA01 on denitrifier community structure showed distinct manners between host strains (**Fig. 4.4A, Fig. 4.4B**; PERMANOVA pairwise, P = 0.002). In Aiptasia CC7 with symbiont SSA01, it was reflected in the enrichment of the predominant taxon 'unclassified bacterial symbiont of *Nonionella stella*' (ASV2, ASV130, ASV299). In contrast, in Aiptasia H2 with symbiont SSA01, this difference was driven by diverse enriched taxa, such as ASV6 (Gammaproteobacteria, *Kangiellaceae*, *Kangiella*), ASV50 and ASV63 (Alphaproteobacteria, *Nitrobacteraceae*, *Bradyrhizobium)*, and ASV58 (unclassified Gammaproteobacteria).

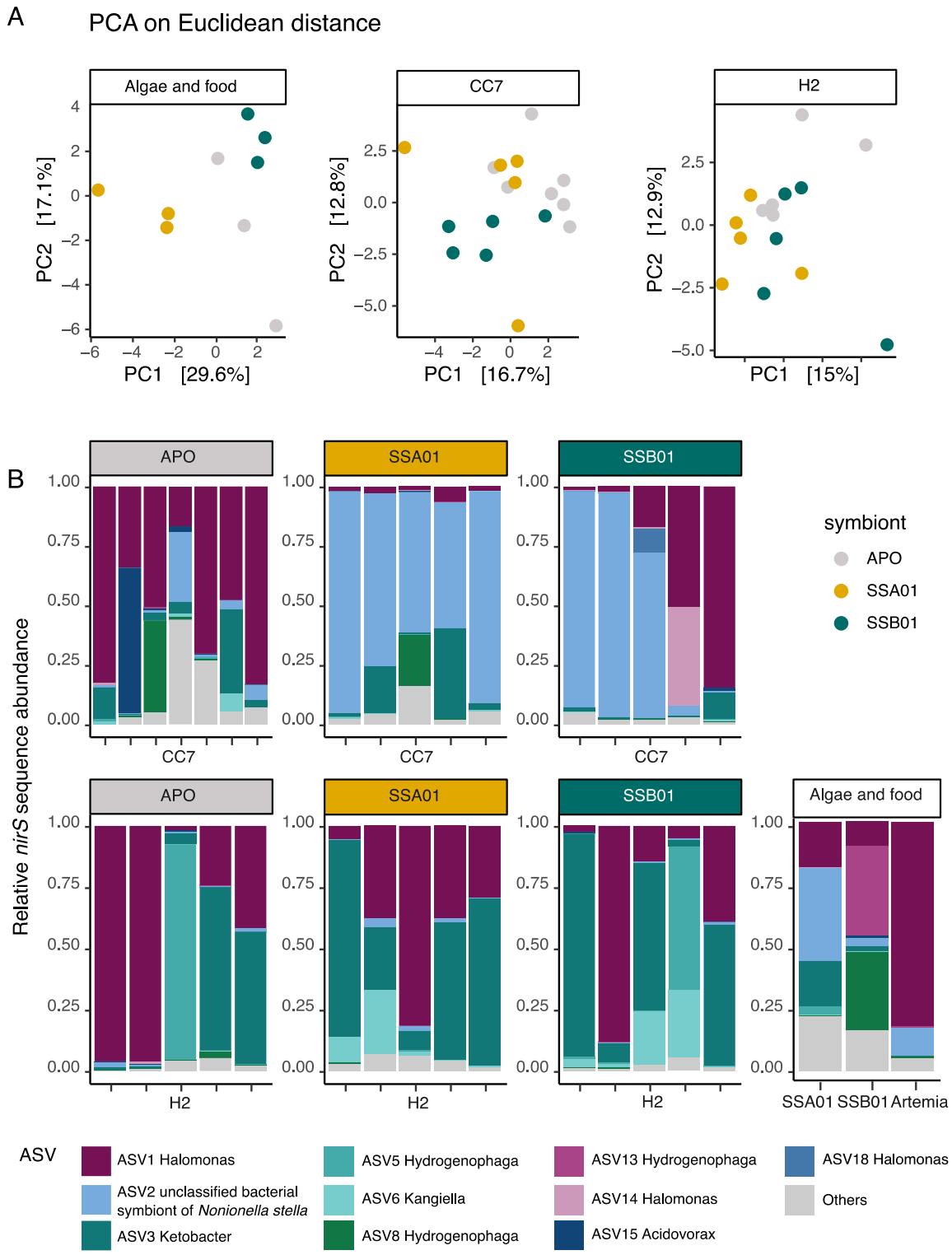

Fig. 4.4 Denitrifier community composition based on *nirS* gene sequencing. A Principal component analysis (PCA) plots of denitrifier community compositions associated with Aiptasia, algal cultures, and food Artemia based on Euclidean distances. **B** Stacked bar plots showing denitrifier community compositions in Aiptasia, algal cultures, and food Artemia. Stacked bar plots display the 10 most abundant bacterial amplicon sequence variants (ASVs).

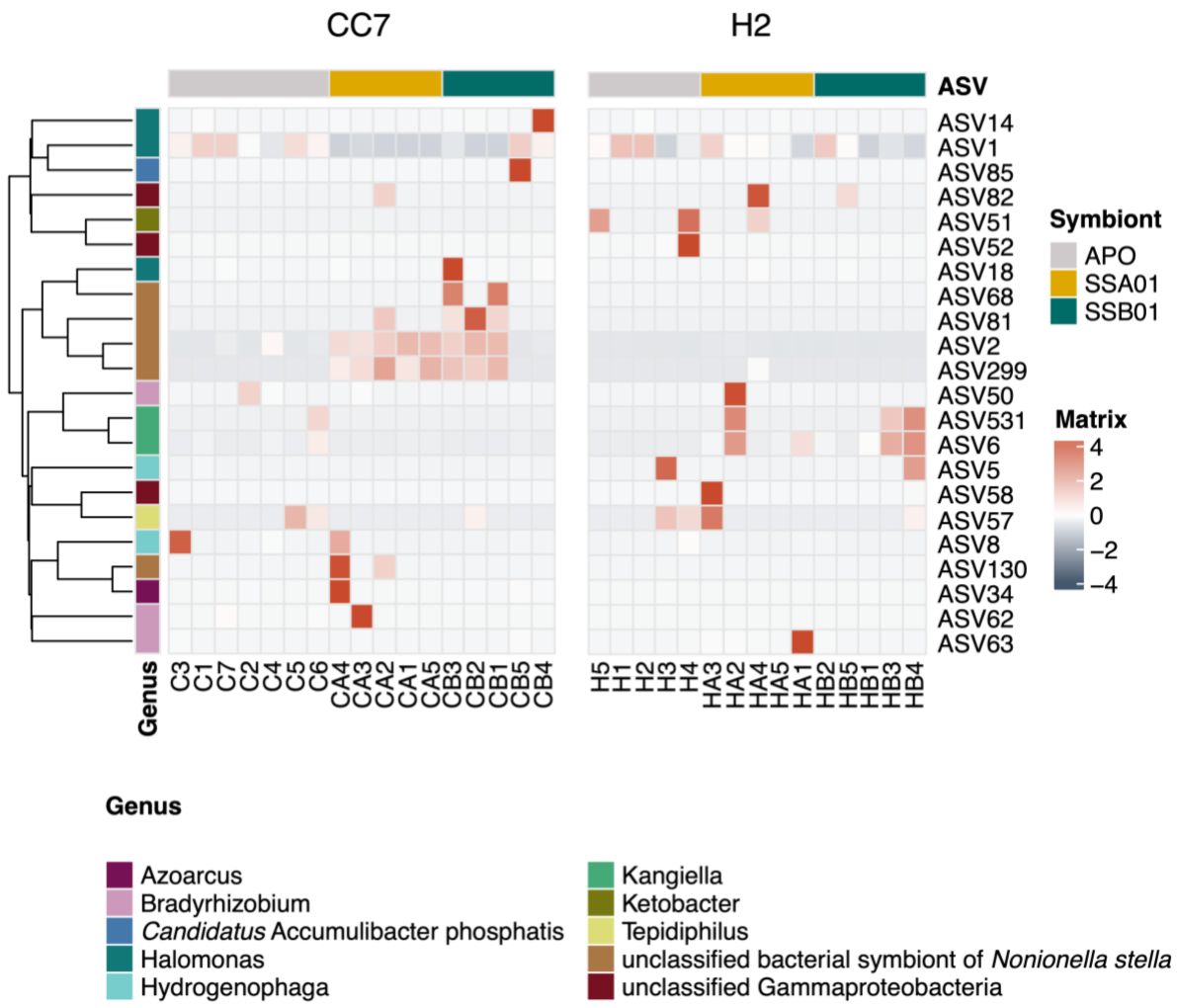

Fig. 4.5 Differential enrichment of denitrifier taxa across six host-symbiont combinations of Aiptasia. Heatmap showing the significantly enriched amplicon sequence variants (ASVs) across samples.

4.5 Discussion

Nitrogen cycling microbes are considered key players in the coral holobiont due to their potential role in stabilizing nitrogen limitation, critical for the functioning of the coral-algal symbiosis (10, 16, 17, 21, 26). To disentangle the putative direct or indirect contribution of the cnidarian host and algal symbionts in the structuring of denitrifier communities, here we quantified the abundance and characterized the community composition of denitrifiers in two Aiptasia strains in an aposymbiotic or symbiotic state with two different Symbiodiniaceae clonal lineages. We found that the presence of algal symbionts strongly increased the denitrifier abundance in Aiptasia, while the denitrifier community composition was jointly affected by host and algal identity. As denitrifier abundance aligned with the photosynthetic carbon

availability of algal symbionts, our findings suggest that denitrifier communities may be passively regulated by the nutritional status of holobionts and could thereby contribute to maintaining a nitrogen-limited state.

4.5.1 Diet-derived denitrifiers dominate *nirS* communities of aposymbiotic Aiptasia

To the best of our knowledge, this study is the first next-generation sequencing characterization of the *nirS*-based denitrifier communities in a photosymbiotic cnidarian. Our results reveal that Aiptasia harbors distinct denitrifier communities with low phylogenetic diversity, dominated by a few bacterial taxa affiliated to Alpha- and Gammaproteobacteria. Thereby, our findings resemble patterns previously reported for diazotroph community compositions in corals, characterized by a high dominance of only a few taxa and similar taxonomy at the Class level (9, 58, 59).

Similar to previous reports (60), artificially bleached Aiptasia still harbored a background community of algal symbionts. However, the low symbiont densities in these animals (orders of magnitude lower than in fully photosymbiotic animals) and the successful replacement of the dominant algal symbionts in symbiotic animals suggest that these animals can be considered functionally aposymbiotic for the purpose of this study. Previous studies reported high rates of inorganic nitrogen release in aposymbiotic Aiptasia due to host catabolic activity (36). While previous studies suggested that these conditions might favor the growth of denitrifiers (61), aposymbiotic Aiptasia showed the lowest relative abundance of denitrifiers among six different host-symbiont combinations in the present study.

This finding is further supported by the strong dominance of ASV1 *Halomonas* in denitrifier communities of aposymbiotic Aiptasia from both clonal lineages. As ASV1 accounted for ~80% of *nirS* sequences associated with *Artemia* nauplii used for feeding of the animals, the dominance of this ASV in denitrifier communities of aposymbiotic animals likely reflects the passive uptake of diet-associated denitrifiers (35). Coupled with the absence of host lineage-specific community differences in aposymbiotic animals, our results suggest that denitrifier communities are extremely rare in the aposymbiotic Aiptasia holobiont and largely reflect the opportunistic acquisition and diet by the host. The low denitrifier abundances might imply reduced microbial nitrogen transformation processes in aposymbiotic Aiptasia (35). Consequently, denitrifiers are unlikely to play an important role in the nitrogen cycle of aposymbiotic cnidarian holobionts.

4.5.2 Presence of algal symbionts promotes denitrifier abundance in photosymbiotic Aiptasia

Increased denitrifier abundances in photosymbiotic Aiptasia compared to their aposymbiotic counterparts were accompanied by altered denitrifier community structures. These findings align with the relative depletion of sequences affiliated to ASV1 *Halomonas* (presumably diet-derived) in photosymbiotic animals, indicating that the denitrifier proliferation may be driven by the onset of the cnidarian-algal symbiosis. Interestingly, the changes in denitrifier abundance and community composition showed clear symbiont-specific patterns. Aiptasia with symbiont SSA01 not only exhibited the highest relative abundances of denitrifiers, but also harbored more differently enriched denitrifier taxa compared to their aposymbiotic or SSB01 symbiotic counterparts. Specifically, Aiptasia with symbiont SSA01 were enriched in denitrifiers assigned as unclassified bacterial symbiont of the foraminifera *Nonionella stella* (consisting of ASV2 and ASV299). As these denitrifiers also dominated the *nirS* community of SSA01 algal cultures, our results suggest that the denitrifier community shifts observed here may, in part, reflect the colonization of the holobiont by algal-associated bacteria (62, 63). In line with this**,** Aiptasia with symbiont SSB01 showed similar, albeit less pronounced, increases in denitrifier abundance and specific enrichment of rare denitrifying taxa (e.g., ASV85 *Candidatus* Accumulibacter phosphatis) compared to aposymbiotic animals.

While the presence of algal symbionts caused pronounced shifts in denitrifier abundances and community compositions of Aiptasia, patterns of community change also depended on the host identity. The denitrifier community in strain CC7 with symbiont SSA01 was dominated by the unclassified bacterial symbiont of *Nonionella stella* (ASV2, ASV130, ASV299) compared to their aposymbiotic counterparts. In contrast, Aiptasia H2 with symbiont SSA01 was enriched in *Kangiella* (ASV6, ASV58) and *Bradyrhizobium* (ASV50, ASV63) compared to aposymbiotic counterparts. It is thus evident that the proliferation of denitrifiers following the onset of the cnidarian-algal symbiosis either favors different algal-associated bacteria depending on host identity or that rare denitrifiers present in aposymbiotic hosts colonize the holobiont in an opportunistic manner. The functional importance of the distinct bacterial community between photosymbiotic and aposymbiotic Aiptasia is currently unclear (61, 64). Here, our finding supports the notion that the putative functional importance of denitrifiers in nutrient cycling is likely limited to the photosymbiotic holobiont, and their community structures depend on both host and symbiont types.

4.5.3 Denitrifier communities reflect the nutritional status of holobionts

There are many environmental drivers that affect denitrifying bacterial activity, such as nitrogen, oxygen, and organic carbon availability (32). However, the abiotic and biotic drivers of the denitrifier community in the cnidarian holobiont remains unknown. In line with previous reports (28, 31), our findings suggest that the availability of nitrogen and oxygen are unlikely major driving forces for cnidarian-associated denitrifiers. The symbiosis of cnidarian host and algal symbionts and concomitant increase in photosynthate availability are known to drastically alter the nutrient availability in the cnidarian holobiont (35, 36). Specifically, the efficient recycling of organic and inorganic carbon in the symbiosis causes a proportional reduction in nitrogen availability in the photosymbiotic holobiont (35, 36). The enhanced oxygen and reduced nitrogen availability in photosymbiotic holobionts should thus indicate unfavorable conditions for denitrifiers (32). However, we observed a pronounced proliferation of denitrifiers following the onset of the cnidarian-algal symbiosis. Interestingly, a majority of denitrifiers detected in photosymbiotic Aiptasia (e.g., *Kangiella*, *Bradyrhizobium* and *Candidatus* Accumulibacter phosphatis) are heterotrophic bacteria (65-67). In line with this, previous studies suggested that the release of photosynthetically-fixed carbon from Symbiodiniaceae may promote bacterial denitrification in the cnidarian holobiont (28).

Importantly, the presence of symbiont SSA01 caused the most pronounced effects on denitrifier communities in both Aiptasia CC7 and H2 holobionts. Concurrently, SSA01-symbiotic holobionts showed consistently higher photosynthetic efficiency and symbiont densities than their SSB01-symbiotic counterparts. Coupled with the higher C:N ratios in SSA01-symbiotic holobionts, our results suggest that SSA01 shows superior *in hospite* physiological performance and causes a more pronounced alteration in the nutrient cycling of Aiptasia holobionts. It is thus plausible that changing patterns in denitrifiers in the present study may largely reflect the opportunistic growth of bacteria in response to the enhanced carbon availability of photosymbiotic holobionts. In this scenario, the specific effect of host and symbiont identity on denitrifier communities may thereby reflect the nutritional consequences of the distinct photo-physiological performance of respective host-algal symbiont combinations (36, 68, 69).

Patterns in the denitrifier community (and by extension, denitrification activity) as consequences of holobiont carbon availability have important implications for the functioning of cnidarian-algal symbiosis. In this scenario, abundant algal photosynthate release might

indirectly promote the opportunistic growth of heterotrophic denitrifiers. Resulting increases in denitrifier activity would, in turn, limit holobiont nitrogen availability for algal symbionts, thereby stabilizing the nutrient limited conditions required for efficient carbon recycling in the holobiont (7, 8, 26). This positive feedback loop between photosynthate release and denitrification may effectively contribute to maintaining the cnidarian-algal symbiosis in a stable holobiont state. At the same time, such dependence of denitrifiers on organic carbon availability may limit their potential contribution to holobiont acclimatization and adaptation (5, 15). As decreases in algal photosynthate release under ocean warming (8) would directly reduce denitrifier abundance (hence, denitrification activity) (28), it is unlikely that denitrifiers can effectively contribute to maintaining nitrogen limitation at ecologically and physiologically relevant scales in a disturbed cnidarian holobiont.

4.6 Conclusion

The global decline of coral reefs urges the need for a better understanding of holobiont functioning and assembly. Here we show that the onset of the cnidarian-algal symbiosis alters *nirS* denitrifier abundance and community composition in the coral model Aiptasia. Changing patterns in denitrifier communities align with the physiological performance of algal symbionts within their respective host. As such, denitrifier community compositions likely reflect the nutritional status of holobionts. Potentially passively regulated by organic carbon availability, denitrifiers might contribute to maintaining the nitrogen limitation in a stable cnidarian holobiont. Metagenomic and metatranscriptomic studies between photosymbiotic and aposymbiotic Aiptasia, in conjunction with culture-dependent microbial work targeting denitrifiers may further help provide insight into the functional attributes of their respective microbial communities.

4.7 Data Accessibility

Raw *nirS* sequencing data are available on NCBI under BioProject PRJNA836569. The bioinformatic workflow for sequence analysis and data visualization can be assessed at https://github.com/NancyXiang/nirS-Sequence-Analysis.

4.8 Author Contributions

NX, NR, CP, and CRV designed and conceived the research; NX, CP, and NR performed the experiment and analyzed samples; AG and CRV contributed Aiptasia and algal cultures, analytical reagents, and sequencing cost; NX and AC analyzed sequencing data; NX conducted data visualization and statistical analysis; NX, NR, and CP wrote the paper with improvement suggestions from all of co-authors. All authors gave final approval for publication.

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4.10 Supplementary materials

4.10.1 Supplementary Figures

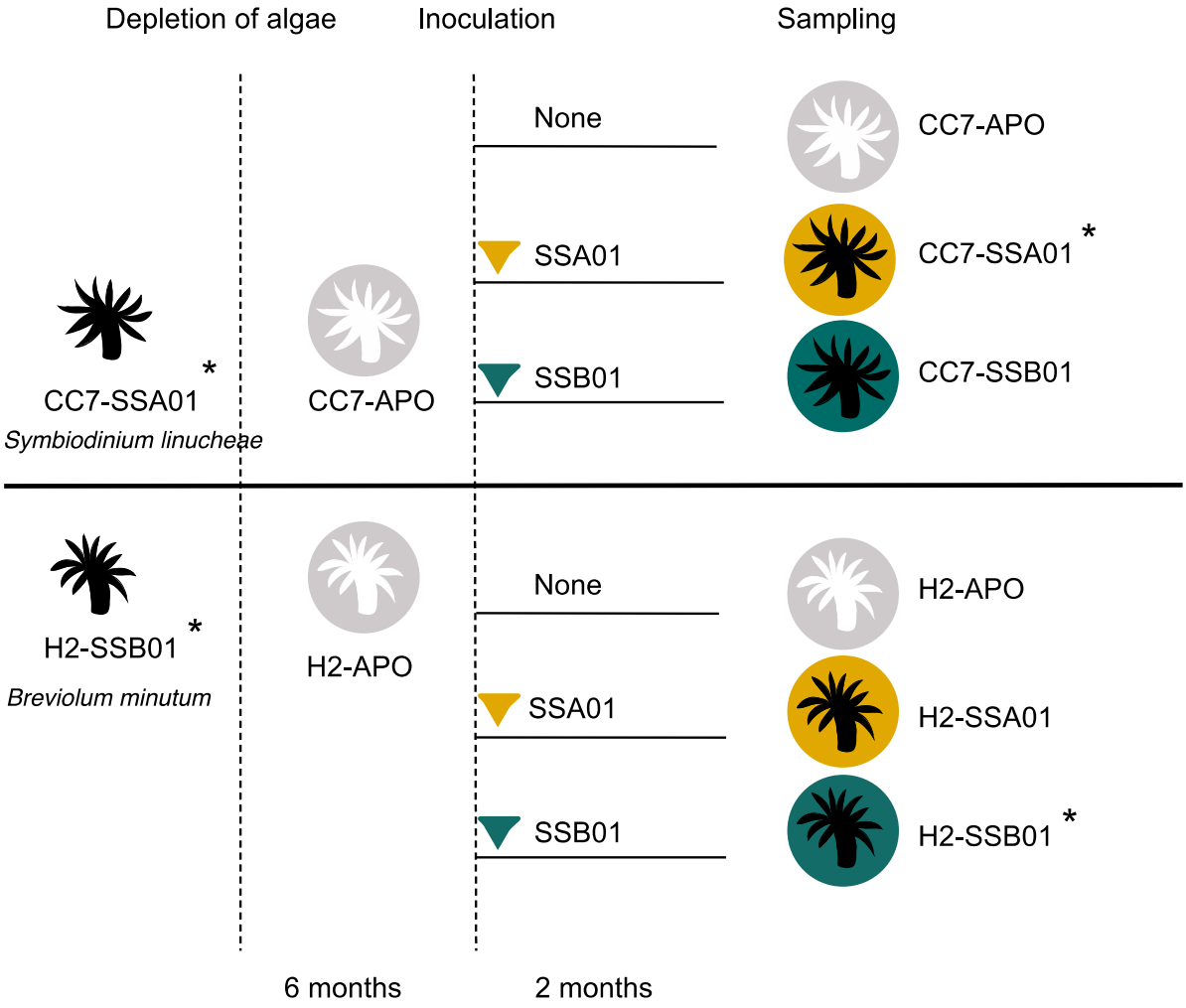

Fig. S4.1 Overview of aposymbiotic Aiptasia generation, algal inoculations and sampling. Aiptasia strains CC7 and H2 were inoculated with two strains of Symbiodiniaceae: *Symbiodinium linucheae* strain SSA01 and *Breviolum minutum* strain SSB01. The native host-symbiont combinations of Aiptasia are indicated by asterisks (*).

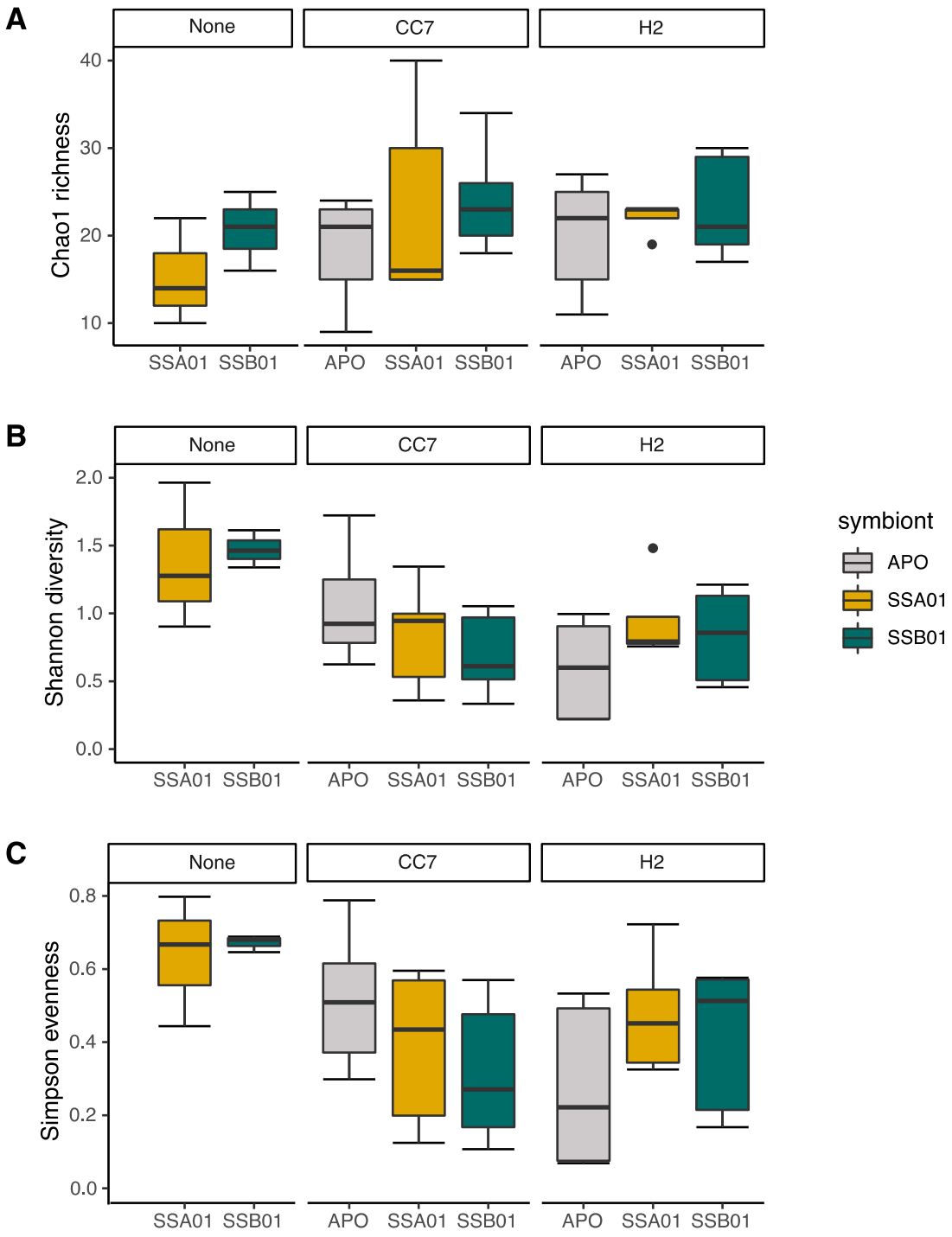

Fig. S4.2 Alpha diversity of denitrifier communities in Aiptasia animals, Symbiodiniaceae, and food samples based on *nirS* gene sequencing. A Chao1 richness. **B** Shannon index. **C** Simpson evenness.

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Chapter 5 General Discussion

5 General discussion

5.1 Overview

Constant and limited nitrogen availability is the foundation for a stable coral-algal symbiosis, thus nitrogen cycling microbes are considered key players in stabilizing or destabilizing coral holobiont functioning (1-8). In this context, it is of utmost importance to assess changes in nitrogen cycler communities in response to environmental change for a better understanding of holobiont functioning. In reef-building corals, global warming and local DOC loading were shown to simultaneously promote the abundance and activity of diazotrophs, resulting in excess nitrogen stress conditions in the holobiont (9-12). For octocorals however, common and abundant benthic reef organisms (13-15), it remained unknown how their associated diazotrophs (if present) respond to environmental disturbances. In addition, denitrifiers were proposed to play a critical role in helping maintain the nitrogen limitation of algal symbionts via active nitrogen removal, thereby contributing to stabilizing the symbiotic relationships (5, 7). However, the abiotic and holobiont-associated biotic drivers of denitrifier abundance and community structure of Cnidaria were largely unknown. In this thesis, we hence provided the first assessment of diazotroph and denitrifier community dynamics in two octocoral species under excess DOC, and combined excess DOC and heat stress over the course of a 6-week aquarium experiment (**Chapter 2, 3**). Furthermore, we disentangled the direct and indirect effect of cnidarian host and algal identity on the structuring of denitrifier communities in the Aiptasia holobiont, a laboratory model system for corals (**Chapter 4**).

In Chapter 2, we found that diazotroph abundance in octocorals was host species-specific and aligned with the trophic strategy and morphology of host animals. These findings are consistent with what has previously been reported for diazotrophs in several species of reef-building corals (16). In contrast to reef-building corals however, diazotroph communities in octocorals did not appear to be sensitive to excess DOC alone, and only proliferated under excess DOC and concomitant warming, which may in part explain the observed high abundance of octocorals on eutrophic reefs (15). In Chapter 3, we found that the overall bacterial community composition of octocorals showed contrasting host-specific dynamics under excess DOC and concomitant warming, with inferred denitrifier abundances having decreased in *X.*

umbellata, but seemingly unaffected in *P. flava*. The prevalent putative denitrifier taxa *Ruegeria*, *Dinoroseobacter*, *Ralstonia*, and *Labrenzia* were previously identified as part of the core microbiome of dinoflagellates (in particular the family Symbiodiniaceae). Finally in Chapter 4, we demonstrated that the presence of algal symbionts boosted denitrifier abundance in the Aiptasia holobiont. The denitrifier community structure in the Aiptasia holobiont was influenced by both host and algal identities, indicating that it may be passively regulated by the nutritional status of the holobiont. In conclusion, the results of this thesis support the importance of nitrogen cycling microbes in the functioning of coral holobionts, and shed light on the putative interactions of Symbiodiniaceae and nitrogen cycling microbes in the coral holobiont.

5.2 Diazotrophs in octocorals (Hypothesis 1)

*Research hypothesis 1: Similar to reef-building corals, excess DOC as readily accessible energy promotes diazotroph abundance in octocorals (**NO**). Concomitant heat stress facilitates the stimulating effect of excess DOC on diazotroph proliferation (**YES**).*

5.2.1 Octocoral diazotroph abundance aligns with trophic strategy and morphology

Diazotrophs have been found in a broad range of reef-building coral species with diverse trophic strategies and taxonomic groups (1, 3, 4, 9, 11, 16-19). With the presence of diazotrophs in the soft coral *X. umbellata* and the gorgonian *P. flava* in this work, we have demonstrated that diazotrophs are members of the octocoral holobiont, regardless of the host's trophic strategy or reef-building capability (1, 3, 4, 9, 11, 16-21). The abundance of diazotrophs was host-specific in the two investigated octocorals, and seemingly aligned with host trophic strategy, as reflected by the higher abundance in the highly autotrophic *P. flava* compared to the mixotrophic *X. umbellata* (20). This finding is consistent with previously reported patterns of diazotroph abundance in reef-building corals (16), suggesting that diazotroph activity may be an additional source of nitrogen for autotrophic corals to compensate for their poor heterotrophy (16, 20, 21).

Apart from their apparent alignment with the trophic strategy of their host animals, it is worth noting that the distinct diazotroph abundance between two investigated octocorals may in part be related to the behavior and morphology of the host species. The pulsation behavior of the soft coral *X. umbellata* provides various benefits to the holobiont, such as enhanced oxygen levels and facilitation of nutrient fluxes (22, 23). However, diazotroph communities are extremely sensitive to oxygen and elevated nutrient levels (5, 24). As a result, the benefits generated by the pulsation behavior of *X. umbellata* could potentially restrict the distribution, abundance, and activity of diazotrophs in the holobiont. This finding is congruent with previous reports showing that diazotroph activity in *Xenia* sp. was 3-fold lower than in the soft coral *Sarcophyton* sp. in the northern Red Sea (25). The relatively higher diazotroph abundance in *P. flava* may be also explained by its axial calcium carbonate skeleton, which is absent in *X. umbellata*. Previous studies have suggested that endolithic prokaryotes constituted a major component of diazotroph communities in reef-building corals (26-28). Likewise, the axial calcium carbonate skeleton of *P. flava* may serve as a suitable habitat for anaerobic diazotrophs, as reported for the skeletons of reef-building corals (26-30, 63).

Prior research has found that diazotroph activity in octocorals was 1 to 2 orders of magnitude lower than it in reef-building corals with voluminous skeletons (4, 25). Given the previously reported positive correlation between diazotroph abundance and activity in the reef-building coral holobiont (16), the diazotroph abundance in octocorals is likely far lower than in reef-building corals. Consequently, the responses of octocoral holobionts to excess DOC or heat stress may differ to those of reef-building corals, where stimulated diazotrophs contributed to disrupting the nitrogen limitation and imperiling the coral-algal symbiosis (9, 10). Hence, the effects of excess DOC and heat stress on octocoral holobionts warrant further investigation. We will hence discuss the diazotroph dynamics in octocorals under environmental change in the followingt paragraph.

5.2.2 DOC boosts diazotroph abundance in heat-stressed octocorals

Excess DOC at 26°C caused no effect on the abundance of diazotrophs in the two investigated octocoral species. Assuming that an absence of change in diazotroph abundances translates into stable diazotroph activity as suggested previously (16), this likely suggests that

excess DOC may not disrupt the nitrogen-limited status (and by extension symbiotic nutrient exchange) of the octocoral holobiont. Indeed, 21 days of excess DOC caused no effect on the phenotype (i.e., no bleaching, tissue loss or host mortality), photosynthesis, or respiration rates of the two octocoral species (31, 32, 64). At this point, it remains unknown why the diazotroph abundance in octocorals did not respond to excess DOC, which contrasts with the previously reported stimulated diazotroph abundance and activity in reef-building corals under excess DOC (10 mg L^{-1}) (9). Such increases in diazotroph abundance and activity have been linked to increases in nitrogen availability in the reef-building coral holobiont, potentially contributing to bleaching and symbiotic breakdown (9). As such, our finding may partly explain why octocorals are more resistant than reef-building corals under organic eutrophication (13-15, 33-36).

Of note, in combination witht heat stress (a stepwise increase to 32^{o}C over 24 days), excess DOC (above 20 mg glucose L^{-1}) promoted a 6- and 4-fold increase of diazotroph abundances in *X. umbellata* and *P. flava*, respectively. In this light, similar mechanisms of symbiotic breakdown in nutrient exchange linked to increased diazotroph activity observed in reef-building coral holobionts may be in place in symbiotic octocoral holobionts (9, 11, 37-39). Thereby, improving water quality by e.g., lowering the DOC loading in local reef regions may effectively help alleviate the excess nitrogen stress in octocoral holobionts in future ocean warming scenario. However, increases in diazotrophy in octocorals may be of limited consequence if compensated by increases in denitrification (5, 7, 40, 41). In the next section, we will thus discuss how the denitrifier communities are distributed among two investigated octocoral species and how they respond to environmental change.

5.3 Denitrifiers in octocorals (Hypothesis 2)

*Research hypothesis 2: Excess DOC and its interaction with heat stress promote denitrifier abundance to counterbalance the elevated diazotroph-derived nitrogen availability in the octocoral holobiont (**NO**). The increasing denitrifier abundance is driven by a few denitrifier taxa that are distinct in different octocoral holobionts (**NO**).*

5.3.1 DOC does not boost denitrifier abundance in heat-stressed octocorals

Denitrifiers were detected in both octocoral species with host-specific abundance by using qPCR. Overall, relative denitrifier abundance was higher in the more autotrophic gorgonian coral *P. flava* compared to the more mixotrophic soft coral *X. umbellata* (20, 21). As more mixotrophic corals may be able to acquire more nitrogen via heterotrophic feeding, this observation indicates that highly autotrophic corals exhibit a greater dependence on nitrogen cycling microbes to regulate the nutrient status of holobionts (16, 20, 21). Similar to diazotrophs, most denitrifiers are anaerobic heterotrophs (42). Thereby, increased oxygen availability generated by the pulsation behavior of *X. umbellata* may also inhibit the growth of denitrifiers in the holobiont (22, 23).

Similar to the diazotroph responses discussed above, excess DOC at ambient temperatures had no effect on denitrifier abundance in two investigated octocoral holobionts. However, under heat stress, excess DOC (above 20 mg glucose L^{-1}) decreased denitrifier abundance in *X. umbellata* by 2- to 3-fold, yet did not alter denitrifier abundance in *P. flava*. Our findings suggest a contrasting microbiome response between diazotrophs (increased abundance as shown in Chapter 2) and denitrifiers (host-specific unaltered or decreased abundance as shown in Chapter 3 here) in octocorals under excess DOC and concomitant heat stress. Consequently, the reduced or unchanged denitrifier abundances are unlikely to buffer the potentially elevated nitrogen availability induced by the increased diazotroph abundance (and therefore activity) in two octocoral holobionts (**Fig. 5.1**). Ultimately, such an imbalance in microbial nitrogen cycling may contribute to the loss of nitrogen limitation in the octocoral holobiont, thereby destabilizing symbiotic nutrient exchange in the octocoral-algal symbiosis during heat stress (9, 11, 37, 38, 43).

Notably, only *P. flava* exhibited bleaching response under excess DOC (above 20 mg glucose L^{-1}) and concomitant heat stress, as characterized by a substantial reduction in the symbiont density and gross photosynthetic activity (64). On the other hand, gross photosynthetic and respiration activities of *X. umbellata* were significantly reduced in the presence of excess DOC and concomitant heat stress, compared to undisturbed conditions (i.e., before the start of experiment), though no mortality or visible bleaching was observed (31, 32).

Thereby, our findings are consistent with the previous studies showing remarkable resistance of *X. umbellata* to environmental disturbances (22, 23, 25, 44). Yet, the role of nitrogen cycling microbes in the bleaching response of octocorals will require further investigation.

5.3.2 Non-complex and similar denitrifier communities in two octocoral holobionts

Denitrifier abundance was distinct between two octocoral species with different trophic strategies. Yet, the community structure and composition of putative denitrifiers in two octocorals was similar and of low phylogenetic diversity, dominated by a few bacterial taxa affiliated to Alpha-, Gamma- and Betaproteobacteria. This finding resembles patterns previously reported for diazotroph community compositions in several reef-building corals, characterized by the dominance of only few taxa and similar community composition at the Class level (11, 18, 19). The non-complex and similar denitrifier communities in two octocorals are largely driven by the high dominance of a putative denitrifier genus *Ruegeria* (Alphaproteobacteria), accounting for more than 90% of the total denitrifier community.

Notably, while denitrifier abundance in octocorals did not respond to excess DOC, the community structure of denitrifiers exhibited a substantial shift. This was reflected in a rapid decrease in the abundance of the predominant *Ruegeria*, which was accompanied by the increase in the relative abundance of several other putative denitrifying genera, including *Dinoroseobacter* in *X. umbellata*, and *Labrenzia* and *Ralstonia* in *P. flava*, respectively. Such community shifts might be explained by differential dependence of denitrifier taxa on labile carbon sources. For instance, some pelagic Rhodobacteraceae taxa dominate the microbial glucose uptake in coastal North Sea waters (45), while others are suppressed by the allochthonous glucose loading (46). At this point, the causes and consequences of the altered denitrifier community structure for octocoral holobiont fitness and functioning remain to be determined.

Interestingly, all four putative denitrifiers *Ruegeria*, *Dinoroseobacter*, *Labrenzia*, and *Ralstonia* were previously identified as members of the core microbiome of Symbiodiniaceae (47-52). *Ruegeria* (formerly named *Silicibacter*) are dinoflagellate-associated bacteria capable

of catabolizing DMSP (an osmolyte and powerful scavenger of reactive oxygen species) produced by the algal host (47). Some *Labrenzia* have previously been reported to produce DMSP and thereby potentially contribute to the reduction of oxidative stress of Symbiodiniaceae in the coral holobiont (48, 50). *Dinoroseobacter* could provide the dinoflagellate host with vitamins B_1 and B_{12}, to support their growth and metabolism (51). Finally, *Ralstonia* occurring within the endodermal coral host cells in close proximity to the Symbiodiniaceae have been proposed to be implicated in the health of the coral-algal symbiosis (52), although specific functions have yet to be identified. These findings strongly suggest that there may be intimate interactions between denitrifiers, Symbiodiniaceae, and the coral host. Therefore, in the next section, we will discuss the potential role of the host and algal identities in structuring denitrifier communities in the cnidarian holobiont.

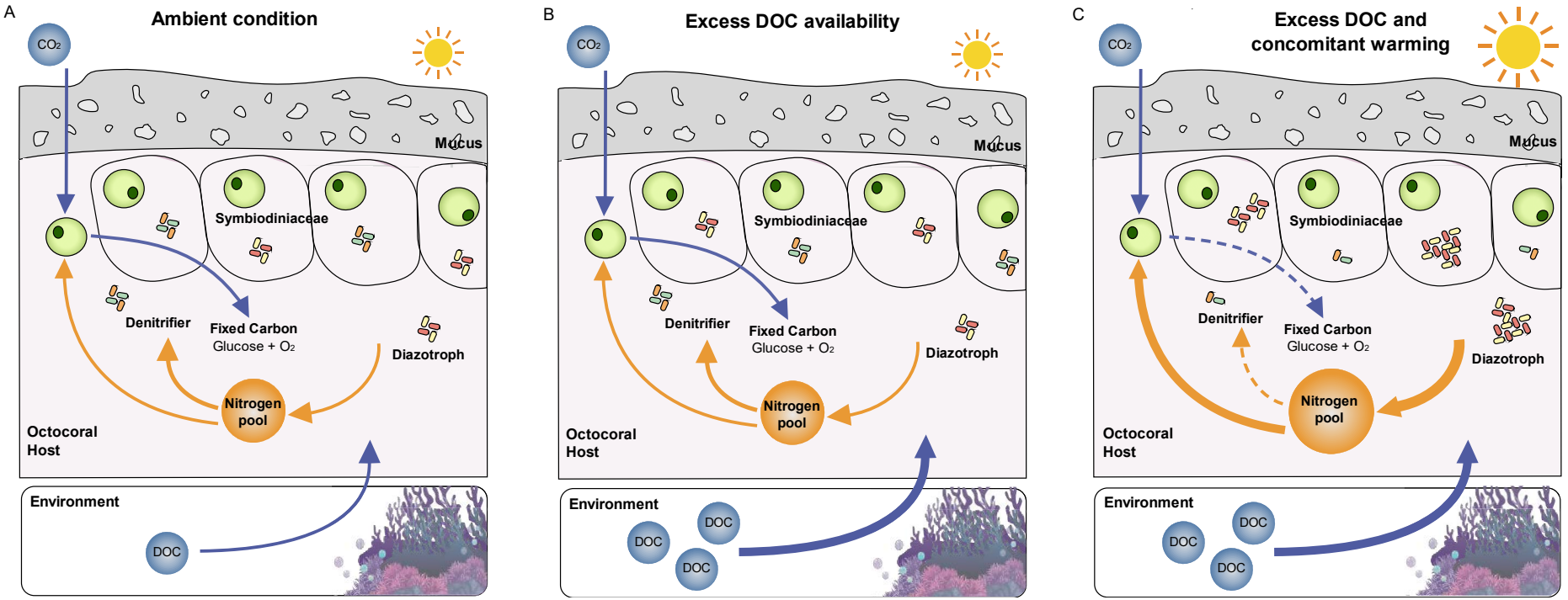

Fig. 5.1 Conceptual overview of the effect of excess DOC and concomitant warming on microbially-mediated nitrogen cycling process in the octocoral holobiont. A Octocorals under undisturbed (ambient) conditions. **B** Octocorals exposed to excess DOC. **C** Octocorals exposed to excess DOC and concomitant warming. Created by Nan Xiang (conceptualization) and Susana Simancas (visualization).

5.4 Presence of algal symbionts increases abundance and alters community structure of denitrifiers (Hypothesis 3)

*Research hypothesis 3: The presence of algal symbionts promotes denitrifier abundance in the cnidarian holobiont (**YES**).*

The sea anemone *Exaiptasia diaphana* ("Aiptasia") is an emerging model organism to study the coral-algal symbiosis, because of its unique advantage of being amenable to manipulations of endosymbiotic algal communities (53-55). Using a high-throughput *nirS* sequencing approach, here we have shown that Aiptasia-associated denitrifier communities were with low phylogenetic diversity, dominated by a few bacterial taxa belonging to Alpha- and Gammaproteobacteria (Chapter 4). These findings resemble patterns in diazotroph community compositions in reef-building corals (11, 18, 19), as well as patterns inferred by the functional attribution for octocoral-associated denitrifier communities (Chapter 3).

Denitrifier communities in both aposymbiotic Aiptasia strains, CC7 and H2, were dominated by *Halomonas* (Gammaproteobacteria) making up ~90% of the overall denitrifier community. As *Halomonas* also accounted for ~80% of the denitrifier community in brine shrimp nauplii used for feeding Aiptasia, we hypothesize that denitrifiers in aposymbiotic animals may be primarily derived from their diet. In this light, the denitrifier abundances in aposymbiotic Aiptasia appears to be low, suggesting low microbial nitrogen transformation potential in these holobionts (55). As a result, denitrifier communities are unlikely to play a critical role in the nitrogen cycling of aposymbiotic cnidarian holobionts.

Following inoculating two aposymbiotic host strains with the Symbiodiniaceae strain SSA01 (*Symbiodinium linucheae*) cultures, total denitrifier abundances quantified by qPCR increased by 4-fold and 22-fold in Aiptasia CC7 and H2, respectively. Following inoculation and colonization of hosts with SSA01, a rapid increase in denitrifier taxa was observed. Yet, their taxonomic identity was also dependent on the identity of the host. As these emerging taxa were also found in SSA01 cultures, we thus hypothesize that the observed rise in denitrifier abundance in the photosymbiotic holobiont is likely driven by the colonization of denitrifier

taxa associated with Symbiodiniaceae cultures which were used for inoculation of host animals (48, 50). This is consistent with our prior findings (in Chapter 2) that the dominant putative denitrifiers in octocorals are likely composed of members of the Symbiodiniaceae core microbiome (47-52).

Similarly, the inoculation with the Symbiodiniaceae strain SSB01 (*Breviolum minutum*) cultures onto aposymbiotic Aiptasia animals also increased denitrifier abundance (2-fold for CC7 and 3-fold for H2, respectively) and shifted their overall community structure. Yet, the effects of SSB01 inoculation on denitrifier community patterns in Aiptasia holobionts were less pronounced than the inoculation with SSA01. Inoculation with SSB01 resulted in distinct increases of individual denitrifier taxa between two Aiptasia host strains. It remains unknown why the same algal strains (SSA01 or SSB01) inoculated onto two different aposymbiotic Aiptasia host strains (CC7 and H2) resulted in the differential establishment of various denitrifier taxa. However, this finding may indicate that the denitrifier community pattern in the cnidarian holobiont may not only be related to the presence and identity of algal symbionts but also to the environment provided by the host animals (54, 55).

Taken together, we showed that the presence of algal symbionts resulted in increased denitrifier abundance in the Aiptasia (by extension cnidarian) holobiont, but at different orders of magnitude in the two Aiptasia strains. Therefore, the denitrifier community structure in the Aiptasia holobiont is apparently influenced by both host and algal identities. Thereby, patterns in the denitrifier community may reflect emerging physiological properties of distinct host-symbiont configurations, such as nutritional status and oxygen availability (5, 55). In the next section, we will hence discuss the potential linkage between denitrifier communities and the nutritional status of cnidarian holobionts.

5.5 Denitrifier community reflects the nutritional status of holobionts (Hypothesis 4)

*Research hypothesis 4: Denitrifier community in the cnidarian holobiont is highly dominated by a few heterotrophic prokaryotes (**YES**), thus photosynthetic carbon availability is one of the main driving forces for denitrifier patterns (**YES**).*

The establishment and observed shifts in the denitrifier community following the onset of the Aiptasia-algal symbiosis have important ecological implications. Previous studies have shown that the Aiptasia host controls endosymbiotic algal densities by regulating nitrogen availability in the holobiont (55, 56). Our findings tentatively suggest that denitrifiers may also contribute to the maintenance of nitrogen limitation and thereby the control of algal populations in the stable cnidarian symbiosis. In comparison to inoculation with strain SSB01, the inoculation with strain SSA01 onto the aposymbiotic Aiptasia resulted in more significant alterations in denitrifier abundance and community structure. At the same time, we found that Aiptasia holobionts infected with algal strain SSA01 exhibited a higher photochemical efficiency than holobionts infected with algal strain SSB01 both in homologous (CC7) and heterologous (H2) hosts. This aligns with prior studies that SSA01 exhibits a superior photosynthetic performance than SSB01 (57). The enhanced photosynthetic activity of SSA01 algae enhances labile carbon avilability in the holobiont, which may cause a more pronounced shift in the nutritional status of other holobiont members including denitrifiers.

Interestingly, the prevalent (putative) denitrifiers in Aiptasia (*Kangiella*, *Bradyrhizobium*, and *Candidatus* Accumulibacter phosphatis) and octocoral holobionts (*Ruegeria*, *Labrenzia*, *Dinoroseobacter*, and *Ralstonia*) are all heterotrophic bacteria (47-52, 58-59). In this light, organic carbon released by algal photosynthesis may serve as an important energy source for the heterotrophic denitrifiers. Such nutritional regulation of denitrifiers in the cnidarian holobiont could directly explain the positive correlation of denitrifier abundance and Symbiodiniaceae density observed in reef-building corals (60).

The passive regulation of denitrifier communities by algal photosynthetic carbon availability in the cnidarian holobiont has important ecological implications. Here, I propose a positive feedback loop of Photosynthetic carbon, Denitrifier, Nitrogen limitation, and Symbiosis (PDNS) in the photosymbiotic cnidarian holobiont (**Fig. 5.2**). This positive feedback

loop between photosynthate release and denitrification may effectively contribute to maintaining the cnidarian-algal symbiosis in the unperturbed (steady state) holobiont. Abundant photosynthetic organic carbon released by algal symbionts in the intact symbiosis might support the growth of heterotrophic denitrifiers. Denitrifier populations, in turn, help maintain the nitrogen limitation for algal symbionts via nitrogen removal, thereby helping control algal density and stabilizing symbiotic nutrient exchange in the cnidarian holobiont (37-39).

However, this system may be delicate and easily disturbed by global and local environmental change. The algal density and photosynthetic carbon availability in cnidarian holobionts may rapidly decrease in response to various environmental disturbances such as ocean warming and eutrophication (9, 38, 39, 61, 62). In this scenario, decreases in photosynthetic carbon availability may result in decreases in the denitrifier abundance and activity, thereby effectively undermining their proposed function of maintaining nitrogen limitation in the coral holobiont (5). As such, a dependence of denitrifiers on photosynthetic carbon availability might limit their potential to contributing tomaintaining nitrogen limitation at ecologically and physiologically relevant scales in a disturbed cnidarian holobiont. This finding may help explain the unchanged or even reduced denitrifier abundance in octocorals under DOC and concomitant heat stress (Chapter 3). In conclusion, our findings show that denitrifiers may be crucial partners in supporting cnidarian holobiont stability and functioning. However, in the face of global environmental change, the potentially beneficial role of denitrifiers may be hampered due to their dependence on photosynthetic carbon availability in the cnidarian holobiont.

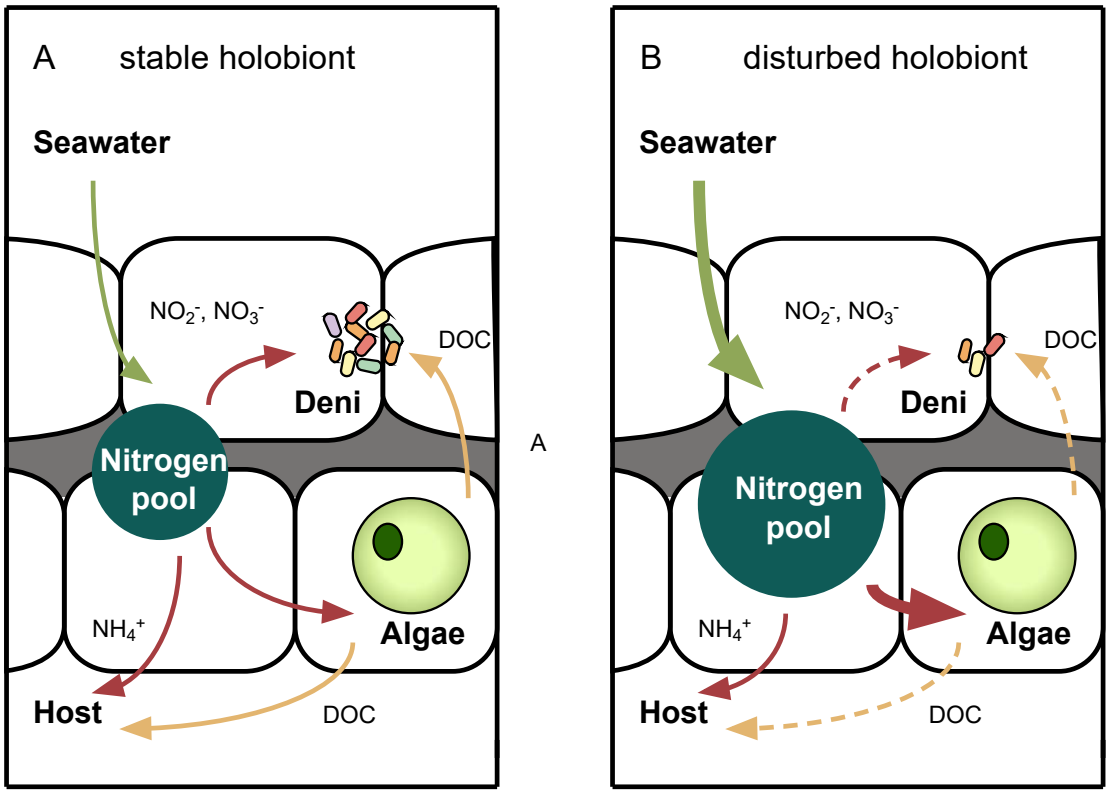

Fig. 5.2 Conceptual model of the positive feedback loop of Photosynthetic carbon, Denitrifier, Nitrogen limitation, Symbiosis (PDNS) in the photosymbiotic cnidarian holobiont. A In the stable cnidarian holobiont. **B** In the disturbed cnidarian holobiont with reduced algal density and photosynthetic carbon availability, induced by multiple global and local environmental stressors (e.g., ocean warming, organic and inorganic eutrophication) (9, 11, 38, 39, 61, 62). Abbreviation: Deni = Denitrifying prokaryotes; DOC = Dissolved organic carbon; NH_4^+ = Ammonia; NO_3^- = Nitrate; NO_2^- = Nitrite. Adapted from Rädecker et al., (2021) (11). Created by Nan Xiang.

5.6 Conclusion and future outlook

This thesis confirmed the presence of two major nitrogen cycling microbial functional groups, specifically diazotrophs and denitrifiers, in two species of octocorals with contrasting trophic strategies: the soft coral *Xenia umbellata* and the gorgonian *Pinnigorgia flava*. Consistent with previously reported patterns of diazotroph abundance in reef-building corals, diazotroph and denitrifier abundances in octocorals are also host-specific and align with the trophic strategy and morphology of the host. Unlike diazotrophs in reef-building corals, the diazotrophs and denitrifiers in octocorals appear to be more resistant to DOC loading in the absence of heat stress. However, under concomitant heat stress, excess DOC promoted the proliferation of diazotrophs in two octocoral species without a corresponding increase in the abundance of denitrifiers, which may result in excessive nitrogen availability that may ultimately threaten the octocoral-algal symbiosis. In addition, this thesis has revealed that the presence of algal symbionts increased the abundance of denitrifiers in the cnidarian holobiont, suggesting intricate interactions between algal symbionts and prokaryotes. Specifically, the photosynthetic organic carbon produced by algal symbionts may serve as an important energy source for heterotrophic denitrifier communities. Thereby, this thesis provides important new insights into the intimate interactions between nitrogen cycling prokaryotes, the host, and algal symbionts in the cnidarian holobiont. Importantly, this thesis gives rise to numerous exciting and impactful questions and venues for future research, as outlined below.

First, as the laboratory in which part of this research was conducted is located far away from tropical coral reefs, we were logistically constrained by the number of octocoral species to study. Future research on coral-associated nitrogen cycling should expand to a larger taxonomic framework of octocorals, ideally with a broad range of host taxonomy and life history traits, including but not limited to different trophic or reproductive strategies, or symbiotic status. Further, comparing nitrogen cycling microbial dynamics in a range of marine cnidarian hosts, including octocorals, reef-building corals, or different symbiotic sea anemones in a changing environment may give new insights into the ecological drivers of novel reef ecosystems in which reef-building corals are replaced as dominant ecosystem engineers.

Second, the ability of coral holobionts to maintain the internal nitrogen limitation appears to be important to stabilize the coral-algal symbiosis under environmental change. This thesis focused on denitrifiers with regard to the nitrogen removal process; future studies should take both denitrifiers and anaerobic ammonium oxidation (anammox) prokaryotes into consideration. The role of denitrifiers and anammox prokaryotes in maintaining nitrogen limitation in the coral holobiont needs to be investigated by assessing the microbial activity of both processes with isotope labelling approaches coupled with gene expression analysis of involved microbial pathways. In particular, it would be interesting to check whether the respective contributions of denitrifiers and anammox prokaryotes (if present) in maintaining nitrogen limitation of the cnidarian holobiont would shift under different environmental conditions. Other under-investigated nitrogen cycling pathways, for example, the DNRA (Dissimilatory Nitrate Reduction to Ammonia) process should further be considered in future studies. Coral hosts appear to only take up inorganic nitrogen in the form of ammonia, thus the DNRA may be an important process that could facilitate access of coral hosts to additional nitrogen sources, thereby indirectly controlling the nitrogen availability of algal symbionts.

Third, we found that some of the denitrifiers were partnered with the algal symbionts and present in the cnidarian holobiont following the inoculation with algal cultures. Nitrogen is a limiting but vital element for algal development, thus the relationship between algae and their associated denitrifiers may change depending on the environmental conditions. Within the cnidarian holobiont, denitrifiers may compete with the algal symbionts for nitrogen sources, potentially assisting in maintaining the nitrogen limitation, required for symbiosis maintenance. Future studies should look into the mechanism and eco-evolutionary dynamics of denitrifier-algal competition and its importance during the onset of cnidarian-algal symbiosis. Integrated holistic approaches combining *in situ* measurement of denitrification activity, localization of denitrifiers in the intact symbiosis, and a combination of '-omics' approaches may aid such a challenging endeavor.

Finally, other prospective directions should include cultivation-dependent techniques to obtain genomic information of denitrifier strains isolated from different coral reef cnidarian holobionts, which will also permit microbiome manipulation experiments for mechanistic interrogation of the cnidarian-Symbiodiniaceae-denitrifier relationship. Microbiome

manipulations in particular could be of interest in combination with gene knockouts in selected transformed denitrifier isolates to gain a deeper understanding of the role of nitrogen cycling microbes underpinning the cnidarian-algal symbiosis.

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6 Appendix

6 Appendix

6.1 Molecular identification of octocoral species

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This project was initiated by Nan Xiang (80%) and Dr. Achim Meyer (20%). Prof. Dr. Christian Wild (100%) contributed coral samples. Sample processing was conducted by Nan Xiang (85%) with the assistance of Dr. Achim Meyer (15%). Phylogenetic analysis and data visualization were conducted by Nan Xiang (70%) and Dr. Achim Meyer (30%).

6.1.1 Materials and Methods

6.1.1.1 Coral husbandry and sampling

Octocoral specimens *Xenia* sp. and *Pinnigorgia* sp. are aquarium strains that are cultivated (temperature: 26 ± 0.5 °C; pH 7.8 ± 0.2; salinity: 35 ± 3 ‰; 12 h light and 12 h dark) for more than 2 years at the Marine Ecology Department of the University of Bremen. About 1 g (wet weight) coral sample was cut from each specimen with a scalpel and shock frozen in liquid nitrogen. The samples were grinded without thawing using mortar and pistil and were stored at -80 °C until further processing.

6.1.1.2 DNA extraction and PCR

DNA was extracted using the Quick-DNA Universal Kit Quick Protocol for Solid Tissue (ZYMO RESEARCH, USA) according to the manufacture instruction. The DNA was quantified by spectrophotometry at 260 nm and 280 nm using Infinite 200 PRO (Tecan, Austria) and visually inspected on a 1 % (wt/vol) agarose gel electrophoresis (Biometra Horizon 58, Germany).

Mitochondrial barcoding genes *mutS*, *COI* and nuclear ribosomal 28S rRNA gene regions were amplified using gene-specific primers (**Table 6.1**). The PCR condition for three genes was in a total volume of 22 µL, containing 10.0 µL 2X Taq DNA Polymerase Master Mix (VWR, USA), 0.5 µL $MgCl_2$ (25 mM, VWR, USA), 1.0 µL forward primer (10 µM), 1.0 µl reverse primer (10 µM), 7.5 µL DEPC water (NEB, USA) and 2.0 µL DNA (10 ng μL^{-1}). The PCR program was run with an initial DNA denaturation at 94 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 53 °C for 60 s (*mutS* gene), 60 °C for 90 s (*COI* gene), or 56 °C for 45 s (28S rRNA gene), extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

PCR products were qualified by 1 % (w/v) agarose gel electrophoresis and sent for Sanger sequencing (StarSeq, Germany) after SAP digested (VWR, USA). Sequencing reads were trimmed using the default quality settings in the software Geneious Prime (version 2021.1.1) (1). Forward and reverse reads of each gene were assembled using De Novo mode. The consensus sequences were used as query in BLASTn searches against the nucleotide database in NCBI Genbank. Alignments with 100 % identity sequences with voucher specimen were taken as species names.

Table 6.1 Primer overview for mitochondrial and nuclear ribosomal gene amplifications.

Gene	Nucleotide sequence (5'-3')[a]	Product	Reference
mutS	F: ND42599F (GCCATTATGGTTAACTATTAC)	747 bp	Sánchez et al., 2003
	R: mut3458R (TSGAGCAAAAGCCACTCC)		
COI	F: COII8068F (CCATAACAGGRCTWGCAGCATC)	1100 bp	McFadden et al., 2004
	R: COIOCTR (ATCATAGCATAGACCATACC)		France & Hoover, 2001
28S	F: 28S-C2 (GAAAAGAACTTTGRARAGAGAGT)	800 bp	Chombard et al., 1998
	R: metazoa (CATCGCCAGTTCTGCTTAC)		Voigt et al., 2012

[a] Forward and reverse primers are indicated by the beginning letters F and R, respectively. For the degenerate nucleotide codes, R = A/G; S = C/G; W = A/T.

6.1.2 Results

The mitochondrial barcode *mutS* gene, *COI* gene, and nuclear ribosomal 28S rRNA gene were amplified from two investigated octocoral species. The resultant sequences were 100% identical to the gene-specific sequences of *Xenia umbellata*/*Xenia hicksoni* and *Pinnigorgia flava*, as summarized in **Table 6.2**. The museum vouchers for corals and their-associated gene accessions were shown in **Table 6.3**.

Table 6.2 Molecular identification of octocoral species. The calculated overall mean distance calculated on the trimmed alignment is 0.00.

Coral	Gene	Length (bp)	Species (No of specimen)	Identical base	Mismatch base
Xenia sp.	*mutS*	781	*Xenia umbellata* (11)	718	0
			Xenia hicksoni (2)	718	0
	COI	914	*Xenia umbellata* (9)	767	0
			Xenia hicksoni (2)	830	0
	28S	147	*Xenia umbellata* (8)	147	0
			Xenia hicksoni (2)	147	0
Pinnigorgia sp.	*mutS*	808	*Pinnigorgia flava* (1)	735	0
	COI	942	*Pinnigorgia flava* (2)	888	0
	28S	699	*Pinnigorgia flava* (2)	699	0

For *Xenia* sp., the identical *mutS* sequence (Accession no: ERZ1762409) was 100 % similar to the reference sequences of *X. umbellata* and *X. hicksoni*. The identical *COI* sequence (Accession no: ERZ1762252) was 100% similar to the reference sequences of *X. umbellata* and *X. hicksoni*. The identical 28S rRNA gene sequence (Accession no: ERZ1761210) was 100 % similar to the reference sequences of *X. umbellata* and *X. hicksoni*. For *Pinnigorgia* sp., the identical *mutS* sequence (Accession no: ERZ1762410), *COI* sequence (Accession no: ERZ1762270), and 28S rRNA gene sequence (Accession no: ERZ1761211) were 100% similar to the gene-specific sequences of *P. flava.*

Table 6.3 The museum voucher for corals and their-associated gene accession number. Coral species that were 100% similar to *Xenia* sp. and *Gorgonia* sp. of this study based the identical sequences for three genes.

Species with identical sequence	**Museum voucher**	**Accession *mutS* gene**	**Accession *COI* gene**	**Accession 28S rRNA gene**
Xenia umbellata	ZMTAU:36792	KT590460.1	KT590441.1	MK400153.1
	ZMTAU:36791	-	KT590440.1	KY442370.1
	ZMTAU:37034	KT590459.1	-	-
	ZMTAU:36790	KT590458.1	KT590439.1	KY442369.1
	ZMTAU:36788	KT590457.1	KT590438.1	KY442367.1
	ZMTAU:36883	KT590456.1	-	-
	ZMTAU:36877	KT590455.1	-	-
	ZMTAU:36780	KT590454.1	KT590437.1	KY442359.1
	ZMTAU:36784	KT590453.1	KT590436.1	-
	ZMTAU:36783	KT590452.1	KT590435.1	KY442362.1
	USNM:1202016	KC864921.1	KC864990.1	KY442391.1
	USNM:1202005	KC864912.1	KC864981.1	KM201437.1
Xenia hicksoni	ZMTAU:Co.34073	MK396705.1	MK396749.1	MK400170.1
	ZMTAU:Co.34072	GQ342529.1	GQ342463.1	JX203759.1
Pinnigorgia flava	RMNH:Coel.40990	-	KF955138.1	KF915430.1
	RMNH:Coel.40815	-	-	JX203711.1
	-	-	GQ342422.1	-
	-	GQ342498.1	-	-

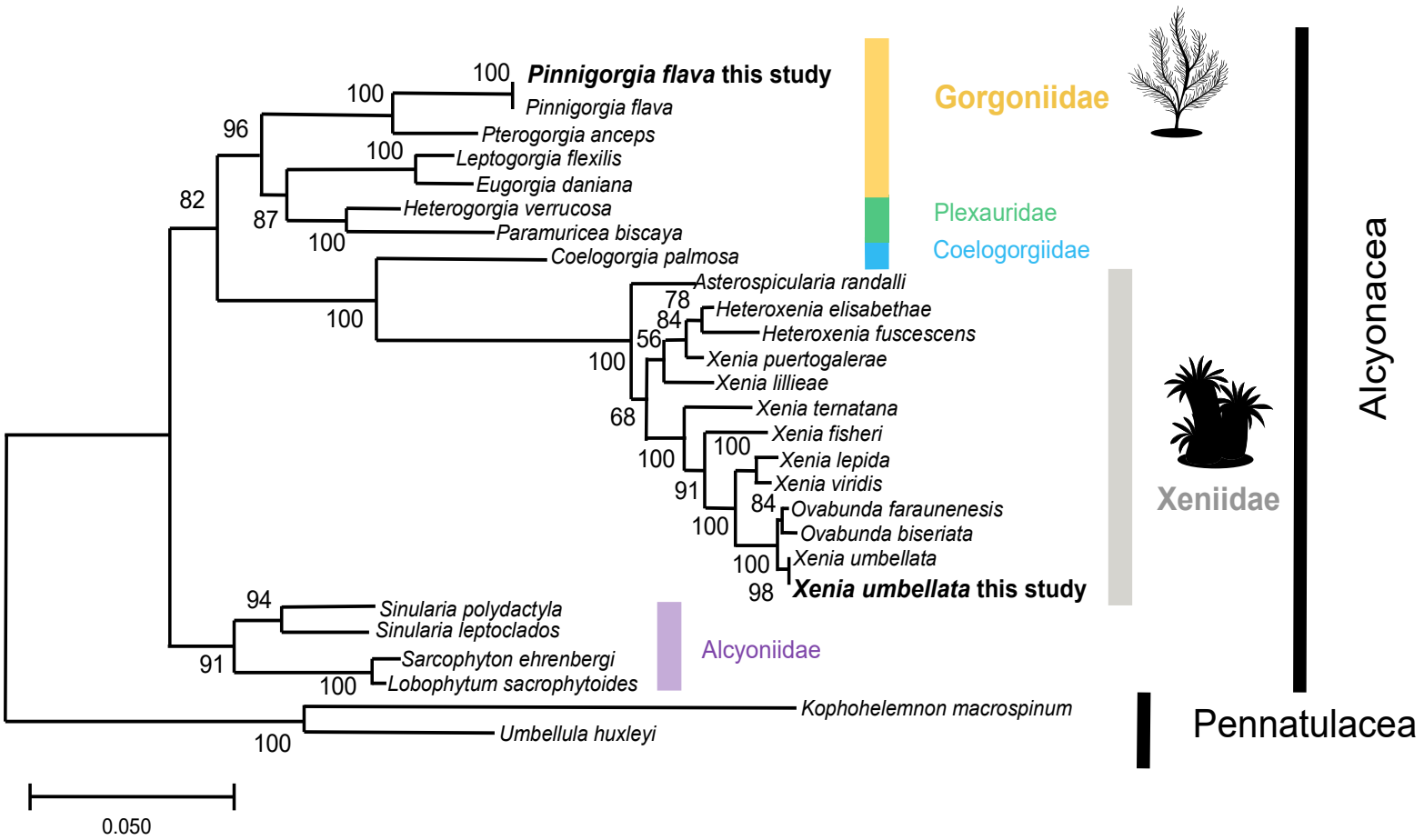

Fig 6.1 Phylogenetic relationship of *Xenia umbellata* and *Pinnigorgia flava* to other Octocorallia. Pennatulacea and Alcyoniidae were used as outgroups. The tree was generated by the neighbor-joining tree building algorithm. Bootstrap values are shown as branch points. Bar: 0.05 nucleotide substitutions per site.

6.1.3 Discussion and Conclusion

The molecular identification of octocoral species suggests *X. umbellata/hicksoni* (3) and *P. flava* (Nutting, 1910) as organisms in this study. This finding aligns with the morphology of both octocoral species (2, 4). Notably, we found that *X. umbellata* and *X. hicksoni* (Ashworth, 1899) shared the same identical sequence in the mitochondrial barcode *mutS* gene, *COI* gene, and nuclear ribosomal 28S rRNA gene. This finding supports the notion of (5), *X. umbellata* and *X. hicksoni* from the Red Sea share a high degree of sequence similarity in mitochondrial barcode genes. A recent study has suggested to include the Red Sea *X. hicksoni* specimens to the group of *X. umbellata* (6). The mother colony of *Xenia* sp. in our study was originally collected from the Red Sea. To this end, we conclude *X. umbellata* as its species name.

6.2 Abstract for co-authored papers during the Ph.D.

6.2.1 Organic eutrophication increases resistance of the pulsating soft coral *Xenia umbellata* to warming

Svea Vollstedt, **Nan Xiang**, Susana Marcela Simancas-Giraldo, and Christian Wild. (2020).

Published in the journal *Peer J*. DOI: https://doi.org/10.7717/peerj.9182.

Abstract

Recent research indicates that hard corals in a process that is termed phase shift are often replaced by soft corals in reefs. The simultaneous occurrence of local (i.e. organic eutrophication as highly under-investigated parameter) and global (i.e. ocean warming) factors may facilitate these phase shifts as hard corals are negatively affected by both ocean warming and organic eutrophication. Knowledge about soft coral responses to environmental change remains incomplete, although these organisms are becoming important players in reefs. The present study thus investigated the individual and combined effects of organic eutrophication (as glucose addition) and warming on the ecological data of the pulsating soft coral *Xenia umbellata*. We assessed health status, growth and pulsation rates of soft corals in a 45 day aquarium experiment, with first manipulation of organic eutrophication (no, low, medium and high glucose addition) over 21 days followed by step-wise increases in water temperature from 26 to 32ºC over 24 days. Findings revealed that glucose addition did not affect health status, growth and pulsation rates of the investigated soft corals. Under simulated ocean warming, soft corals that had experienced organic eutrophication before, maintained significantly higher pulsation rates (averaging 22 beats per minute bpm) and no mortality compared to the controls that showed a decrease of 56% (averaging 15 bpm) in pulsation rates and mortality of 30% at water temperatures of 32ºC compared to 26ºC. This apparently positive effect of organic eutrophication on the ecological data of soft corals under an ocean warming scenario decreased with increasing water temperature. This study thus indicates that (a) organic eutrophication as additional energy source up to a certain threshold may increase the resistance of soft corals to ocean warming and (b) pulsation rates of soft corals may be used as inexpensive, easily detectable, and non-invasive early warning indicator for ocean warming effects on benthic reef communities. When comparing findings of this study for soft corals with previous results for hard corals, it can be assumed that soft corals under the predicted increases of organic

eutrophication and warming gain more and more competitive advantages. This may further facilitate phase shifts from hard to soft corals in warming reefs.

Key words: Soft corals, Local stressor, Global stressor, Organic eutrophication, Warming, Growth rates, Mortality, Pulsation rates

6.2.2 Photosynthesis and respiration of the soft coral Xenia umbellata respond to warming but not to organic carbon eutrophication

Susana Marcela Simancas-Giraldo, **Nan Xiang**, Meghan Moger Kennedy, Rassil Nafeh, Edoardo Zelli, and Christian Wild. (2021).

Published in the journal *Peer J*. DOI: https://doi.org/10.7717/peerj.11663.

Abstract

Eutrophication with dissolved organic carbon (DOC) as a far under-investigated stressor, and ocean warming, can strongly affect coral reefs and hard corals as major reefs ecosystem engineers. However, no previous studies have investigated the metabolic responses of soft corals to DOC eutrophication, or its interaction with ocean warming. Thus, we investigated respiration and photosynthesis response of *Xenia umbellata*, a common mixotrophic soft coral from the Indo-pacific, to (1) three levels of DOC eutrophication simulated by glucose addition over the first 21 days of experiment and (2) ocean warming scenarios where the temperature was gradually increased from 26°C (control condition) to 32°C over another 24 days in an aquarium experiment. We found no significant difference in response to DOC treatments and all corals survived regardless of the DOC concentrations, whilst subsequent exposure to simulated ocean warming significantly decreased gross photosynthesis by approximately 50% at 30°C, and 65% at 32°C, net photosynthesis by 75% at 30°C and 79% at 32°C, and respiration by a maximum of 75% at 30°C; with a slight increase at 32°C of 25%. The ratio between gross photosynthesis and respiration decreased by the end of the warming period but remained similar between controls and colonies previously exposed to DOC. Our findings suggest that soft corals may be more resistant than hard corals to DOC eutrophication and in consequence, may potentially experiment in less magnitude the negative effects of increased temperature or subsequently both stressors. The results of this study may contribute to explain the successful role of soft corals in phase shifts as reported from many coral reefs. Where predicted declines in reef ecosystems health due to increased eutrophication levels can be exacerbated by future warming.

Key words: Octocoral, Pulsating coral, Stress modulation, Global stressor, Local stressor, DOC

6.2.3 Coral holobiont cues prime *Endozoicomonas* for a symbiotic lifestyle

Claudia Pogoreutz, Clinton A. Oakley, Nils R decker, Anny C rdenas, Gabriela Perna, **Nan Xiang**, Lifeng Peng, Simon K. Davy, David K. Ngugi, and Christian R. Voolstra. (2022).

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Abstract

Endozoicomonas are prevalent, abundant bacterial associates of marine animals, including corals. Their role in holobiont health and functioning, however, remains poorly understood. To identify potential interactions within the coral holobiont, we characterized the novel isolate *Endozoicomonas marisrubri* sp. nov. 6c and assessed its transcriptomic and proteomic response to tissue extracts of its native host, the Red Sea coral *Acropora humilis*. We show that coral tissue extracts stimulated differential expression of genes putatively involved in symbiosis establishment via the modulation of the host immune response by *E. marisrubri* 6c, such as genes for flagellar assembly, ankyrins, ephrins, and serpins. Proteome analyses revealed that *E. marisrubri* 6c upregulated vitamin B1 and B6 biosynthesis and glycolytic processes in response to holobiont cues. Our results suggest that the priming of *Endozoicomonas* for a symbiotic lifestyle involves the modulation of host immunity and the exchange of essential metabolites with other holobiont members. Consequently, *Endozoicomonas* may play an important role in holobiont nutrient cycling and may therefore contribute to coral health, acclimatization, and adaptation.

Key words: Coral symbiosis, Endozoicomonas, proteomics, transcriptomics, bacterial isolate, nutrient cycling

6.2.4 Individual and combined effect of organic eutrophication and ocean warming on the ecophysiology of the gorgonian *Pinnigorgia flava*

Edoardo Zelli, Susana Marcela Simancas-Giraldo, **Nan Xiang**, Claudia Dessì, Nadim Daniel Katzer, and Christian Wild. (2022).

In revision in the journal *Peer J*. DOI: https://doi.org/10.1038/s41396-022-01226-7.

Abstract

Dissolved organic carbon (DOC) enrichment and ocean warming both negatively affect hard corals, but studies on their combined effects on other reef organisms are lacking. Gorgonians are likely becoming key players in future reef communities, but they are still highly under-investigated with regard to their responses to global and local environmental changes. Thus, we evaluated the individual and combined effects of DOC enrichment (10 to 40 mg L-1 DOC added as glucose) and warming (from 26 to 32 °C) on the widespread gorgonian Pinnigorgia flava in a 45-day laboratory experiment. Net photosynthesis, respiration, as well as zooxanthellae density and growth were assessed over time. Our results showed that gorgonian ecophysiology responded differently to DOC enrichment and warming as well as their combination. DOC addition did neither significantly affect gorgonian photosynthesis and respiration activity nor zooxanthellae density and growth. But warming significantly decreased photosynthesis activity and zooxanthellae density. When DOC eutrophication and warming were combined, coral growth significantly decreased, while no effect on photosynthesis, respiration, and zooxanthellae density was observed. Our findings indicate that the gorgonian Pinnigorgia flava is likely more resistant than hard corals to the single effect of organic eutrophication, but not to ocean warming. This may contribute to explain the previously observed competitive advantage of gorgonians over hard corals in coastal reef ecosystems that are often affected by DOC eutrophication.

Key words: Coral Reefs, Octocorals, Photosynthesis, Respiration, Organic Carbon Enrichment, Global Warming

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Ort, Datum: Bremen, 01 July 2022

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Ich versichere an Eides Statt, dass ich die vorgenannten Angaben nach bestem Wissen und Gewissen gemacht habe und dass die Angaben der Wahrheit entsprechen und ich nichts verschwiegen habe.
Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahren Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs. 1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

Bremen, 01 July 2022

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Ort, Datum Unterschrift