Benthic dinitrogen fixation in a Northern Red Sea coral reef under seasonally changing environmental conditions

Dissertation

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Dedicated to my parents

"All life is part of a complex relationship

in which each is dependent upon the others,

taking from, giving to and living with all the rest."

Jacques-Yves Cousteau

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Zusammenfassung

Tropische Korallenriffe besiedeln die nährstoffärmsten Gewässer, gehören jedoch weltweit zu den produktivsten Ökosystemen. Um diese enorme Produktivität zu erzielen ist ein effektives Recycling der limitierenden Nährstoffe, insbesondere Stickstoff (N), notwendig, und zusätzlich der Eintrag von biologisch verfügbarem N über den Prozess der Stickstofffixierung (N_2-Fixierung). Verschiedene benthische Organismen und Substrate sind mit einer diversen Gemeinschaft von diazotrophen (N_2-fixierenden) Mikroorganismen assoziiert, jedoch ist der jeweilige Beitrag der einzelnen benthischen Gruppen zur N_2-Fixierung des gesamten Riffbenthos und der Effekt von Umwelteinflüssen bislang nicht untersucht worden. In der vorliegenden Doktorarbeit wurde in einem stark saisonal geprägten Meeresgebiet, dem nördlichen Roten Meer, in einer ganzen Reihe von miteinander verbundenen Studien benthische N_2-Fixierung untersucht, um neue Erkenntnisse über diesen Prozess unter sich verändernden Umweltbedingungen in Korallenriffen zu erlangen. Diese Arbeit hatte folgende zugrundeliegende Schlüsselfragestellungen:

1) Wieviel N_2 wird von den dominanten benthischen Gruppen im nördlichen Roten Meer fixiert?
2) Was ist der relative Beitrag der einzelnen benthischen Gruppen zu der gesamten benthischen N_2-Fixierung im Korallenriff?
3) Welchen Einfluss haben saisonal bedingte Umweltschwankungen sowie einzelne Umweltfaktoren auf Stoffwechselprozesse, speziell auf N_2-Fixierung, der untersuchten benthischen Gruppen?

Die Ergebnisse zeigten, dass alle untersuchten benthischen Gruppen diazotrophe Aktivität aufwiesen, wobei Korallengesteine, filamentöse Algen, Karbonatsande und lebende Steinkorallen mit ~ 90 % den Hauptanteil der benthischen N_2-Fixierung im untersuchten Korallenriff ausmachten. Weichkorallen fixierten vergleichsweise weniger N_2 und gaben zusätzlich signifikant weniger organisches Material als Steinkorallen ab, wodurch die Funktion von Korallen als allogener

Ökosystem Ingenieur möglicherweise von N_2-Fixierung beeinflusst wird. Generell wurde unter geringer Nährstoffverfügbarkeit sowie maximaler Wassertemperatur und Lichteinstrahlung am meisten N_2 fixiert, was daraufhin deutet, dass fixierter N vor allem unter nährstoffarmen Bedingungen (z.B. in den Sommermonaten) für den Stoffwechsel der Rifforganismen von entscheidender Bedeutung ist. Die Manipulation einzelner Umweltfaktoren deutete an, dass globale Ozeanerwärmung die Fixierung von N_2 erhöhen kann, während Ozeanversauerung sie erniedrigt. Somit könnte der Nährstoffhaushalt von Rifforganismen durch den Klimawandel deutlich beeinflusst werden.

Schlüsselergebnisse dieser Doktorarbeit sind zusammenfassend, dass N_2-Fixierung durch benthische Rifforganismen und Substrate allgegenwärtig ist, und dass der N_2-Fixierung dabei offensichtlich eine Schlüsselrolle im Steuern der Produktivität von Korallenriffökosystemen zukommt. Hierbei haben sowohl die Zusammensetzung der benthischen Riffgemeinschaft als auch die vorherrschenden Umweltbedingungen Einfluss auf die Quantität der N_2-Fixierung. Da Korallenriffe vermehrt globalen und lokalen Stressfaktoren ausgesetzt sind, ist es notwendig zu verstehen inwieweit wichtige Stoffwechselprozesse (z.B. N_2-Fixierung) sich in Korallenriffen verändern, womit die vorliegende Arbeit aus physiologischer, biogeochemischer und ökologischer Sicht wichtige neue Erkenntnisse liefert.

Summary

Tropical coral reefs are among the most productive ecosystems on this planet, despite being surrounded by very oligotrophic waters. Effective recycling processes of the limiting nutrients, particularly nitrogen (N), and input of new bioavailable N *via* dinitrogen (N_2) fixation are essential to sustain such high gross primary production. In fact, several benthic reef organisms and substrates are associated with diverse communities of N_2 fixing microbes (diazotrophs), but the respective contribution of the different benthic groups to total benthic N_2 fixation and the effect of changing environmental conditions on N_2 fixation have not been investigated yet. Therefore, this thesis, through a series of interconnected studies carried out in a seasonally dynamic coral reef system, the Northern Red Sea, and in a number of manipulative experiments, contributes to the understanding of benthic N_2 fixation in coral reefs by answering the following key questions:

1) How much N_2 is fixed by the dominant benthic groups in the Northern Red Sea?
2) What is the relative contribution of the benthic groups to total benthic N_2 fixation within the reef?
3) What is the effect of seasonally changing environmental conditions and of single environmental factors on key metabolic processes, particularly N_2 fixation, associated with the different benthic groups?

Findings revealed that all investigated benthic groups showed N_2 fixation activity, whereof bare coral rock, turf algae, carbonate sands and living hard corals were the main N_2 fixing components contributing ~ 90% to benthic N_2 fixation in investigated Red Sea coral reefs. Soft corals revealed the lowest N_2 fixation activity among all investigated groups and released significantly less organic matter to the surrounding water compared to hard corals indicating that N_2 fixation may also influence their role as allogenic ecosystem engineers. N_2 fixation by most benthic groups was usually highest when nutrient availability was lowest, and water temperature as well as light intensity highest suggesting that the N_2 fixation products fuel the metabolic N

requirements of reef organisms, particularly during nutrient-depleted conditions (i.e. summer). The manipulation of single environmental factors revealed a stimulation of N_2 fixation activities under global warming conditions and a reduction under ocean acidification scenarios indicating that global climate change will affect the nutrient status of reef organisms.

In summary, this thesis underlines the ubiquity of N_2 fixation associated with different benthic coral reef organisms and substrates, and highlights its importance in sustaining coral reef productivity. Both, the benthic community structure and the prevailing environmental conditions appear to be important in controlling the amount of N_2 fixation in coral reefs. Finally, as coral reefs are increasingly and simultaneously exposed to global and local stressors, it is necessary to understand how important metabolic processes on coral reefs (i.e. organic matter fluxes and N_2 fixation activity) will be affected, and this thesis provides first quantitative insights into these processes from physiological, biogeochemical and ecological points of view.

Chapter I

General Introduction*

Coral Reef Paradox

Warm water coral reefs represent one of the most biodiverse and productive ecosystems on this planet, and they are constrained to the shallow, nutrient-deficient coastal waters of the tropics and subtropics (between 30°N and 30°S) (Odum & Odum 1955, Gattuso et al. 1998). Despite conditions of limited nitrogen (N) availability, the gross primary production of coral reef ecosystems exceeds the production rates of most of the earth's natural ecosystems, both aquatic and terrestrial, a condition considered to be a paradox (D'Elia 1988, Muscatine & Porter 1977). The high productivity of coral reefs cannot be sustained solely through the limited input of nutrients from the surrounding oceans. However at times nutrients can be supplied by upwelling or internal tidal bores (Gattuso et al., 1998), which bring nutrient-rich water from deeper layers to the shallower waters, as well as by nutrient advection or loading from land (Alongi & McKinnon 2005, Lapointe et al. 2004). Therefore, in order to maintain the high levels of productivity observed on coral reefs, efficient recycling of both the limited nutrient and the accumulated organic matter must be maintained in order to persist in oligotrophic waters (O'Neil & Capone 2008). In fact, coral reefs are characterized by an optimized utilization of the nutrients and organic matter, whereby both are recycled and retained in living organisms and/or sediments of the reef system (Suzuki et al. 1995, Wild et al. 2004). However, gross photosynthesis could not exceed respiration if only regenerated nutrients were available, and thus net growth requires the input of new N into the system (Szmant-Froelich 1983). Besides the recycling of organic N compounds, dinitrogen (N_2) fixation by reef-associated microbes represents an important source of new bioavailable N in coral reef ecosystems (Larkum et al. 1988, Shashar et al. 1994a, 1994b, Kneip et al. 2008).

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N_2 fixation

Nitrogen is an essential element for the synthesis of nucleic acids and proteins, the two most important polymers for life. Despite its importance, the largest reservoir of N in the atmosphere and in the oceans is represented by the inert molecule N_2 which is biologically not available for most organisms. Thus, biological N_2 fixation, or the conversion of N_2 into organic N, is the most important source for fixed N, particularly in oligotrophic environments (Gruber et al. 2008). The biochemical process of N_2 fixation is limited to specialized prokaryotes (diazotrophs) that consist of a small, but diverse, group of bacteria and archaea occurring in virtually all ecosystems (Kneip et al. 2007, LaRoche & Breitbarth 2005, Zehr et al. 2003). The nitrogenase enzyme complex catalyzes the reduction of N_2 gas into bioavailable ammonium (NH_4^+), and involves various ATP-generating processes in providing the high activation energy required to break the triple bond of N≡N (LaRoche & Breitbarth 2005):

$$N_2 + 8H^+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16Pi$$

(ATP = adenosine triphosphate, ADP = adenosine disphosphate, Pi = inorganic phosphorus)

The input of fixed N *via* N_2 fixation balances the loss of fixed N *via* denitrification from the system, and thus determines the pool of bioavailable N and overall marine productivity (Gruber et al. 2008). The largest source of fixed N in the oceans is derived from pelagic N_2 fixation with current estimates of around 100 to 150 Tg N $year^{-1}$, followed by input of fixed N from rivers (~ 80 Tg N year-1), from atmospheric deposition (~ 50 Tg N $year^{-1}$), and from benthic N_2 fixation (~ 15 Tg N $year^{-1}$; reviewed in Gruber 2008). Among benthic N_2 fixation in marine environments most of it is fixed in shallow, nearshore areas (Table 1.1).

N_2 fixation in coral reefs

Since the 1970s, studies investigating N_2 fixation of coastal ecosystems concluded that a major fraction of total benthic N_2 fixation may come from shallow environments such as sea grass meadows, coral reefs, salt marshes or mangroves (Capone & Carpenter 1982, Capone 1983; Table 1.1). Estimates showed that coral reefs, along with salt marshes, display the highest N_2 fixation rates of all coastal environments. In fact, N_2 fixation in coral reefs was estimated to provide 2.8 Tg N y^{-1} which accounts to ~18 % of total benthic N_2 fixation in the marine environment. Total benthic N_2 fixation is similar for shelf sediments (2.7 Tg N y^{-1}; 0-200 m depth), however, when considering

fixation per unit area, coral reefs are magnitues higher than shelf sediments (Table 1.1). Such high rates in coral reefs are most likely supported by high light availabilities and effective recycling loops of the scarcely available phosphorus which can often limits N_2 fixation (Wiebe et al. 1975, D'Elia 1988). Also, the recycling of organic matter in coral reefs may support N_2 fixation activity by acting as an energy source for diazotrophs.

Table 1.1. Surface area and estimated annual N_2 fixation rates for benthic marine environments (modified from Capone and Carpenter 1982).

Environment	**Surface area**	**N_2 Fixation**	
	$km^2\ 10^6$	$g\ N\ m^{-2}\ y^{-1}$	$Tg\ N\ y^{-1}$
Estuaries	1.08	0.4 ± 0.07	0.4
Sea grass meadows	0.28	5.5	1.5
Coral reefs	0.11	25 ± 8.4	2.8
Salt marshes	0.26	24 ± 10.5	6.3
Mangrove forests	0.13	11	1.5
0 – 200 m water depth	27	0.1 ± 0.04	2.7
> 200 m water depth	335	0.01	0.2

Benthic diazotrophs in coral reefs grow either as biofilms on substrate surfaces or on living organisms, or they form symbiotic associations with other reef organisms (Fiore et al. 2010). Epibenthic diazotrophs occur in reef sediments, on dead coral skeletons, on coral rubble or on limestone surfaces of coral reefs (Wiebe et al. 1975, Wilkinson et al. 1984, Larkum 1988, O'Neil & Capone 1989, Shashar et al. 1994b, Charpy-Roubaud et al. 2001, Davey et al. 2008, Werner et al. 2008). N_2 fixation by these communities can account for up to 28 % of total N_2 fixation in the entire coral reef environment (Charpy-Roubaud et al. 2001, Charpy-Roubaud & Larkum 2005). N_2 fixation has been further identified in bacterial epiphytes, on benthic reef macroalgae, and in association with algal turfs and seagrass meadows (Williams & Carpenter 1997, 1998, France et al. 1998, Welsh 2000, Koop et al. 2001).

Several benthic reef organisms such as living hard corals (Williams et al. 1987, Shashar et al. 1994, Lesser et al. 2004, 2007, Lema et al. 2012), sponges (Wilkinson & Fay 1979, Mohamed et al. 2008), sea urchins (Guerinot et al. 1981), and ascidians (Welsh 2000, Carpenter & Foster 2003) form symbiotic associations with diazotrophs. In scleractinian corals, endolithic cyanobacteria within the coral skeleton may be responsible for the majority of N_2 fixation (Shashar et al. 1994a).

Recently, N_2 fixing cyanobacteria were also found within the coral tissue of the stony coral *Montastraea cavernosa* (Lesser et al. 2007, Lesser et al. 2004). The presence of diazotrophs within these corals can enhance cell division rates and population sizes of the endosymbiotic zooxanthellae, when compared to corals without diazotrophic associations (Lesser et al. 2007, Olson et al. 2009). Moreover, this is further supported by studies identifying the origin of N in scleractinian corals, through the use of stable isotope anylsis, and found that a high amount of N in the zooxanthellae is derived from N_2 fixation when the coral is associated with diatotrophs (Lesser et al. 2007). Therefore, in the highly N depleted waters that characterize most coral reefs, the presence of diazotrophs that thrive in symbiotic association with corals and their unicellular algae suggests that N_2 fixation may be an important additional source of N within the host. In this multi-partner symbiotic system (holobiont) (Knowlton & Rohwer 2003, Krediet et al. 2013), it is possible that both the animal host and the zooxanthellae benefit from the N fixed by the diazotrophs, while both the coral and the diazotrophic bacteria receive the photosyntates (i.e. any product of photosynthesis) produced by the dinoflagellate algae.

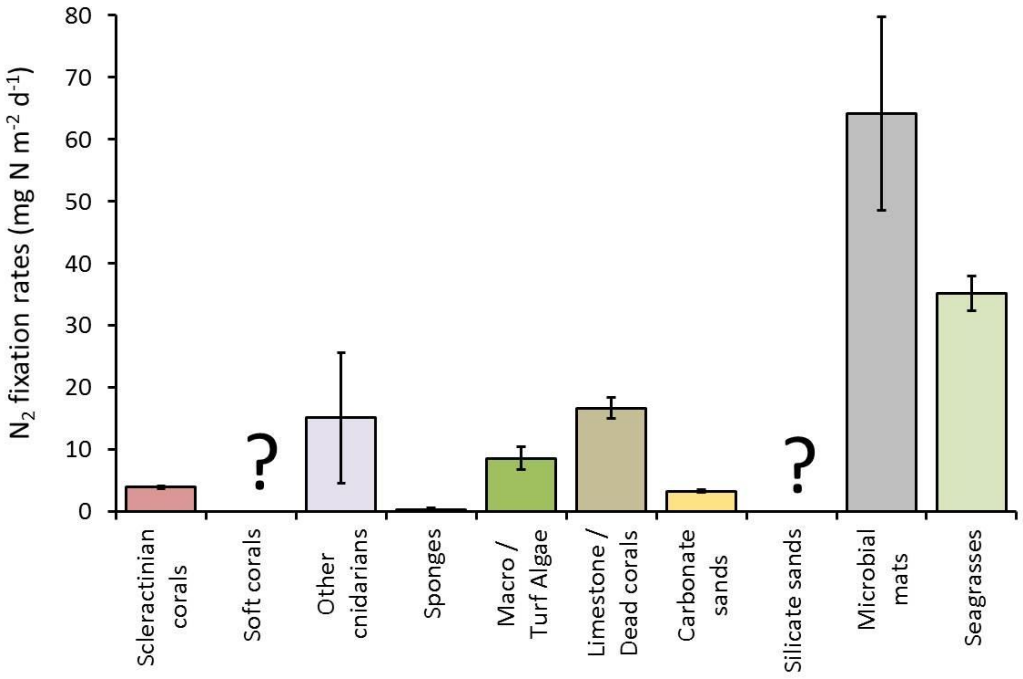

Figure 1.1. N_2 fixation rates (average ± SE) of the main benthic coral reef components. Values were obtained from the available studies which reported nitrogenase activity associated with benthic reef organisms and substrates normalized to surface area. (modified from Cardini et al. 2014).

A comparative overview on N_2 fixation rates associated with the different benthic coral reef organisms and substrates shows that rates of N_2 fixation can be highly variable among the constituents of the coral reef environment (Figure 1.1; modified from Cardini et al. 2014). Microbial mats and seagrasses clearly show the highest N_2 fixation rates per unit surface area when compared to the other benthic groups. N_2 fixation rates of scleractinian corals and reef carbonate sands are comparable, yetslightly lower than rates measured in algae, limestones and dead coral skeletons, while N_2 fixation in soft corals and reef silicate sands has not been investigated yet.

The effect of key environmentl factors on N_2 fixation

Numerous environmental factors (i.e. light, oxygen and nutrient availability, water temperature and pH) can affect the growth and activity of diazotrophs and subsequently the extent of N_2 fixation in an ecosystem (Carpenter and Capone 2008). While most previous studies investigated these effects on natural or cultured populations of the free-living cyanobacterium *Trichodesmium* spp. from the open ocean, the effect on N_2 fixation activities associated with benthic coral reef constituents has received considerably less attention.

Light and oxygen availability

The amount of N_2 fixation by photoautotrophic diazotrophs is directly linked to the energy and reductant provided from photosynthesis and thus, N_2 fixation is indirectly related to light availability (Carpenter & Capone 2008). N_2 fixation activity of *Trichodesmium* spp. is light saturated above 180 μmol quanta m^{-2} s^{-1}, while at photon fluxes greater than 1100 μmol quanta m^{-2} s^{-1} photoinhibition of N_2 fixation occurres (reviewed in Carpenter & Capone 2008). Photosynthetic oxygen (O_2) evolution also affects N_2 fixation activity. Generally, the nitrogenase enzyme is irreversibly inhibited by molecular O_2, and thus low O_2 levels favor nitrogenase activity (Berman-Frank et al. 2003). The coral reef benthos naturally experiences strong shifts in dissolved O_2 concentrations on a diel basis as the community shifts from net photosynthesis during the day to, respiration during thenight. A study from a coral reef platform on the Great Barrier Reef showed that dissolved O_2 concentrations can range from 2.1 mg O_2 L^{-1} after midnight to more than 10.8 mg O_2 L^{-1} (the limit of the instrument) in the early afternoon (Kinsey & Kinsey 1967). Also, the diffusive boundary layer surrounding stony corals can experience extreme diel fluctuations in dissolved O_2 with concentrations varying from supersaturation during the day to anoxia at night

(Kühl et al. 1995, Shashar et al. 1993). Daily O_2 variations and the composition of the diazotrophic population determine the diel pattern of N_2 fixation activity. Populations dominated by heterocystous bacteria show N_2 fixation maxima during mid-day by fixing N_2 in specialized cells (heterocysts) that separate the nitrogenase enzyme spatially from O_2 evolving photosystem II. In contrast, non-heterocystous bacteria exhibit highest N_2 fixation activity at night and thus temporally separate nitrogenase activity from O_2 inactivation.

Water temperature

Increase in temperature generally stimulates enzyme mediated reactions such as N_2 fixation. The distribution of the well-studied tropical diazotroph *Trichodesmium* spp. is limited by the 20 °C isoterm and associated N_2 fixation activity is generally low until water temperature exceeds 20 °C (Carpenter & Capone 2008). Non-heterocystous forms are most abundant among pelagic diazotrophs in warm tropical oceans, while heterocystous forms are rare. The reduced gas solubility and increased respiration rates in warmer waters make the possession of heterocysts under such conditions disadvantageous (Stal 2009). However, heterocystous diazotrophs occur in tropical benthic coral reef environments (Charpy et al. 2012) and may be of particular advantege when diazotrophs live in close association with a photosynthesizing coral reef organisms. In contrast to the tropics, marine diazotrophs are less abundant and less active in cold water areas of high latitude regions (> 50° latitude). Nevertheless, active diazotrophs have been found operating at near freezing temperatures (Bordeleau & Prévost 1994) and at hydrothermal vent fluids (92°C) (Mehta & Baross 2006), thus indicating that the process of N_2 fixation is not intrinsically limited by temperature. As the water temperature co-varies inversely with the nutrient availability in surface waters, the oligotrophic condition in tropical waters may indeed be of greater influence on the distribution and activity of diazotrophs.

Nutrient availability

The high energetic costs associated with N_2 fixation have resulted in the concept that N_2 fixation will be suppressed as soon as fixed N compounds are sufficiently available in the surrounding water. Although, several studies showed that the nitrogenase enzyme can also be active in the presence of elevated fixed N sources (ammonium, nitrate, urea, amino acids, DON) (reviewed in Karl et al. 2002). Other nutrients such as dissolved inorganic phosphate (DIP),

dissolved organic matter (DOM) and trace metals (e.g. Fe, Mo), are mostly limiting N_2 fixation in the open ocean (Arrigo 2005, Kustka et al. 2002, Mills et al. 2004, Moutin et al. 2005, Wu et al. 2000) and may, therefore, stimulate N_2 fixation in coastal areas when supplied from terrestrial sources and anthropogenic inputs. For example, in the Great Barrier Reef lagoon, N_2 fixation by planktonic cyanobacteria (*Trichodesmium*) significantly increased since the 1920s, most likely due to the increased input of river-borne nutrients (e.g. DIP, Fe, DOM) (Bell et al. 1999). Research up to now has largely focused on the effects of elevated nutrient concentrations on N_2 fixation activity by pelagic cyanobacteria (reviewed in Carpenter & Capone 2008), besides for a few studies (Koop et al. 2001, Short et al. 1990). Koop and colleagues (2001) found that the addition of inorganic N had negative impacts on N_2 fixation in reef sediments, while inorganic P addition caused a strong increase in N_2 fixation. They suggested N_2 fixation as a potential biological indicator of nutrient stress in coral reefs, because of the clear and marked response of this variable to increased nutrient treatments. P enrichement also stimulated rhizosphere N_2 fixation in the tropical seagrass *Syringodium filiforme*, along with its growth and biomass (Short et al. 1990). These findings suggest that coral reef ecosystems may ultimately be P limited as N can be replenished *via* N_2 fixation (Eyre et al. 2008).

Ocean acidification

Uptake of CO_2 by the ocean directly alters the seawater carbonate chemistry and results in a reduction in *pH* and carbonate saturation and an increase in dissolved inorganic carbon availability (Caldeira & Wickett 2005). These modifications, collectively referred to as ocean acidification (OA), are predicted to cause multifarious impacts on coral reefs at all levels from the organism to the ecosystem. The physiological effects of increasing pCO_2 on N_2 fixation have, only recently, been realized, but research up to now only focused on planktonic diazotrophs and cultured isolates. Rising pCO_2 had an overall stimulating effect on N_2 fixation by *Trichodesmium* spp. or *Nodularia spumigena* (Barcelos e Ramos et al. 2007, Wannicke et al. 2012), whereas no effects was observed in a natural community of unicellular cyanobacteria (Law et al. 2012). However, in culture, the unicellular cyanobacteria *Crocosphaera watsonii* responded to both light and pCO_2 with a significant negative effect on gross:net N_2 fixation rates (Garcia et al. 2013), implying enhanced cellular retention of fixed N. Recent findings also stressed the role of light intensity in modulating the effects of pCO_2 on the process of N_2 fixation in *Trichodesmium* (Kranz et al. 2010, Levitan et al. 2010), with high irradiances reducing the stimulatory effect of elevated pCO_2 on gross N_2 fixation

(Garcia et al. 2011). This suggests a potentially limited effect of OA on N_2 fixation by similar benthic diazotrophs in light-saturated, coral reef habitats.

Gaps of knowledge

The current literature implies that associations between coral reef organisms and diazotrophs are likely widespread, however N_2 fixation in coral reefs continues to represent an under-investigated research area. When emphasis has been centered on coral reef organisms, most studies have focused on N_2 fixation associated within a single benthic group (i.e. hard corals, macroalgae, microbial mats, coral rock or reef sand). Unfortunatly, methods for the quantification of N_2 fixation has not been standardized and whereby incubation procedures often vary among studies. Moreover, study sites, or reef locations, also differ making it difficult to compare N_2 fixation rates among benthic groups and thus, rendering it difficult to determine the relative contribution of each benthic group to total benthic N_2 fixation within a reef. Furthermore, there is still a lack of available data about N_2 fixation activity associated with important benthic reef constituents, particularly terrigenous silicate sands and zooxanthellate soft corals. Carbonate sands have already shown high N_2 fixation activity within coral reefs, while the often co-occurring silicate sands, that display highly distinct sediment characteristics, have yet to be investigated. Soft corals can reach high abundances at some coral reefs and thus, they may be an important contributer to new N to the reef ecosystem if they are associated with diazotrophs.

Numerous environmental factors (i.e. light, temperature, O_2, trace metal and nutrient availability) are known to affect the growth and activity of diazotrophs. Previous studies investigated the effects of environmental factors mostly on natural or cultured populations of the free-living cyanobacterium *Trichodesmium* spp. from the open ocean (reviewed in Carpenter & Capone 2008), while the potential impact on benthic N_2 fixation in coral reef ecosystems is largely unknown. As coral reefs are increasingly exposed to changing environmental conditions due to anthropogenic disturbances and climate change, the extent of N_2 fixation in coral reefs may also be affected by these climatic changes, suggesting the need for further invesigation.

Aims of this thesis

Given the paucity of information regarding benthic N_2 fixation in tropical coral reefs, the first aim of this thesis is to quantify, with standardized methodology, the N_2 fixation activity of a wide range of differing coral reef benthic groups (i.e. reef sand communities, reef framework substrates, hard corals and soft corals). This will allow for the calculation and comparison of the relative contribution of each group to N_2 fixation, and furthermore, this data can be used to calculate total benthic N_2 fixation for a benthic coral reef community. The second aim of this thesis is to focus on key environmental factors that affect the metabolic processes and the N_2 fixation associated with the different benthic groups. Examination of N_2 fixation was conducted in a seasonally dynamic coral reef system, the Northern Red Sea, and in a series of manipulative experiments, allowing for the evaluation of environmental conditions, and the effects of specific global and local stressors, on variation in N_2 fixation. Lastly, this thesis brings together N_2 fixation information on one of the broadest sets of benthic coral reef organisms. The inclusion of the effects of season and environment on N_2 fixation rates has resulted in a finer temporal and spatial resolution on N_2 fixation. Therefore this thesis provides one of the most dynamic disseminations of N_2 fixation of benthic coral reef organisms, in the literature.

In summary, the overarching research questions of this thesis are:

1) How much N_2 is fixed by the dominant benthic groups in the Northern Red Sea?

2) What is the relative contribution of the benthic groups to total benthic N_2 fixation within the reef?

3) What is the effect of seasonally changing environmental conditions and of single environmental factors on key metabolic processes, particularly N_2 fixation, associated with the different benthic groups?

Approach

This thesis was conducted in collaboration with the Leibniz Center for Tropical Marine Ecology (ZMT), Bremen (Germany), and the Marine Science Station (MSS), Aqaba (Jordan). The primary work was carried out at the Northern Red Sea (Gulf of Aqaba) over all 4 seasons of the year 2013. The Gulf of Aqaba is the northeastern tip of the Red Sea and harbors some of the most northern located (30°N) tropical coral reefs. The high latitude location creates a strong seasonal

pattern in this area resulting in thermal stratification of the water column during summer and deep-water mixing during winter (Silverman et al. 2007, Carlson et al. 2014). These properties make the Gulf of Aqaba a natural laboratory for studying the effect of seasonally changing environmental conditions on N_2 fixation activity associated with benthic reef organisms and substrates. In addition, manipulative experiments were conducted under controlled conditions to investigate the single effects of light, inorganic nutrient availability, water temperature and pH levels on N_2 fixation and other key metabolic processes of coral reef organisms.

Chapter and publication outline

The general introduction, or Chapter I of this thesis, contains parts of a comprehensive literature review on N_2 fixation in coral reefs, and furthermore, provides information on the potential impacts of global and local stressors on the process of N_2 fixation (Publication 1).

Chapters II to V present studies about N_2 fixation activities associated with different benthic coral reef constituents from the Northern Red Sea under seasonally changing environmental conditions. This includes studies on permeable reef sand communities (Publication 2), reef framework substrates (Publication 3), hard corals (Publication 4) and soft corals (Publication 5). Chapters VI to IX focus on the effect of global and local stressors on N_2 fixation, primary productivity and organic matter fluxes associated with hard and soft corals. This includes the effect of inorganic nutrient availability on primary productivity and organic matter fluxes of soft corals (Publication 6), as well as the effect of light availability (Publication 7), elevated water temperature (Publication 8) and reduced pH levels (Publication 9) on primary productivity and N_2 fixation activity of hard corals.

The thesis conclusion, or Chapter X, compiles the data from Chapters II to IX to give an overview of benthic N_2 fixation on the Red Sea coral reef. This has been calculated in order to identify the relative contribution of each benthic constituent to total benthic N_2 fixation within the reef, and furthermore, to evaluate the constituent-specific response of N_2 fixation to changing environmental conditions.

Publication 1)

Ulisse Cardini, **Vanessa N. Bednarz**, Rachel A. Foster, Christian Wild
Benthic N_2 fixation in coral reefs and the potential effects of human-induced environmental change.
This review was initiated by U. Cardini, V. Bednarz and C. Wild and written by U. Cardini with input from all authors. It has been published in Ecology and Evolution (2014).

Publication 2)

Vanessa N. Bednarz, Nanne van Hoytema, Ulisse Cardini, Malik S. Naumann, Mamoon M. D. Al-Rhaidat, Christian Wild
Dinitrogen fixation and primary productivity by carbonate and silicate sand communities of the Northern Red Sea.
This study was initiated by V. Bednarz, U. Cardini, N. van Hoytema and C. Wild. The work was conducted by V. Bednarz, U. Cardini and N. vanHoytema. V. Bednarz analyzed the data and wrote the manuscript with input from all authors. This article is under review at Marine Ecology Progress Series.

Publication 3)

Laura Rix, **Vanessa N. Bednarz**, Ulisse Cardini, Nanne van Hoytema, Fuad Al-Horani, Christian Wild, Malik S. Naumann
Seasonality in dinitrogen fixation and primary productivity by coral reef framework substrates from the northern Red Sea.
This study was initiated by V. Bednarz, U. Cardini, N. vanHoytema and C. Wild. The work was conducted by L. Rix, V. Bednarz, U. Cardini and N. vanHoytema. L. Rix analyzed the data and wrote the manuscript with input from all authors. This article is under review at Marine Ecology Progress Series.

Publication 4)

Ulisse Cardini, **Vanessa N. Bednarz**, Malik S. Naumann, Nanne van Hoytema, Laura Rix, Rachel A. Foster, Mamoon M. D. Al-Rhaidat, Christian Wild
Microbial dinitrogen fixation sustains high coral productivity in oligotrophic reef ecosystems.
This study was initiated by U. Cardini, V. Bednarz, N. vanHoytema and C. Wild. The experimental work was conducted by U. Cardini, V. Bednarz, N. vanHoytema, L. Rix and M. Naumann. U. Cardini and M. Naumann developed the C and N flux model. R. Foster assisted in the design of the acetylene reduction assays. U. Cardini and C. Wild wrote the manuscript with input from all authors. This article is under review at Scientific Reports.

Publication 5)

Vanessa N. Bednarz, Ulisse Cardini, Nanne van Hoytema, Mamoon M. D. Al-Rhaidat, Christian Wild
Seasonal variation of N_2 fixation and O_2 fluxes associated with two dominant zooxanthellate soft corals from the Northern Red Sea.
This study was initiated by V. Bednarz, U. Cardini, N. vanHoytema and C. Wild. The work was conducted by V. Bednarz, U. Cardini and N. vanHoytema. V. Bednarz analyzed the data and wrote the manuscript with input from all authors. This article has been accepted for publication at Marine Ecology Progress Series.

Publication 6)

Vanessa N. Bednarz, Malik S. Naumann, Wolfgang Niggl, Christian Wild
Inorganic nutrient availability affects organic matter fluxes and metabolic activity in the soft coral genus *Xenia.*
This study was initiated by V. Bednarz and C. Wild. The experiments were conducted by V. Bednarz and W. Niggl. V. Bednarz analyzed the data and wrote the manuscript with input from M. Naumann and C. Wild. This article has been published in the Journal of Experimental Biology (2012).

Publication 7)

Vanessa N. Bednarz, Ulisse Cardini, Nanne van Hoytema, Laura Rix, Malik S. Naumann, Mamoon M. D. Al-Rhaidat, Christian Wild
The effect of light availability on dinitrogen fixation associated with scleractinian corals along a depth gradient in the Northern Red Sea.
This study was initiated by V. Bednarz and C. Wild. The work was conducted by V. Bednarz, U. Cardini, N. vanHoytema, L. Rix and M. Naumann. V. Bednarz analyzed the data and wrote the manuscript with input from all authors. This article will be submitted as a note to the Journal of Experimental Biology.

Publication 8)

Ulisse Cardini, Nanne van Hoytema, **Vanessa N. Bednarz**, Laura Rix, Rachel A. Foster, Mamoon M. D. Al-Rhaidat, Christian Wild
Thermal stress impacts all players of the coral-algal-prokaryote symbiosis affecting the resilience of the holobiont to warming.
This study was initiated by U. Cardini and C. Wild. The experimental work was conducted by U. Cardini, V. Bednarz, N. vanHoytema and L. Rix. R. Foster assisted in the design of the acetylene reduction assays. U. Cardini wrote the manuscript with input from all authors. This article will be submitted to the Proceedings of the Royal Society.

Publication 9)

Nils Rädecker, Friedrich W. Meyer, **Vanessa N. Bednarz**, Ulisse Cardini, Christian Wild
Ocean acidification rapidly reduces dinitrogen fixation associated with the hermatypic coral *Seriatopora hystrix.*
This study was initiated by N. Rädecker, F. Meyer and C. Wild. The experimental work was conducted by N. Raedecker and F. Meyer. V. Bednarz and U. Cardini assisted in the design of the acetylene reduction assays. N. Rädecker wrote the manuscript with input from all authors. This article has been published in Marine Ecology Progress Series (2014).

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Chapter II

Dinitrogen fixation and primary productivity by carbonate and silicate reef sand communities of the Northern Red Sea*

Abstract

Permeable sediments are highly bioactive compartments in coral reefs. The associated dense microbial communities sustain fast degradation of organic matter, thereby playing a key role in nutrient recycling within the reef. Besides nutrient recycling, new nutrients (i.e. nitrogen, N) are acquired by dinitrogen (N_2) fixing microbial communities, but knowledge about the influence of sand mineralogy and key environmental factors on this process is scarce. Therefore, this study quantified seasonal N_2 fixation (*via* acetylene reduction) along with gross photosynthesis (*via* oxygen (O_2) fluxes) by adjacent carbonate and silicate sands in a Northern Red Sea coral reef. Findings revealed significantly higher N_2 fixation in carbonate than silicate sands (2.88 and 1.52 nmol C_2H_4 cm^{-2} h^{-1}) and a more pronounced seasonal response in the former likely caused by its higher permeability, grain size and microbial abundance. Ambient light and organic matter availability were the main sand-associated N_2 fixation controlling factors. Carbonate and silicate sands showed similar gross photosynthesis rates (270 and 233 nmol O_2 cm^{-2} h^{-1}) that positively (carbonate sands) or negatively (silicate sands) correlated with N_2 fixation likely due to different diazotrophic communities. Seasonal appearance of microbial mats on carbonate sands increased N_2 fixation and gross photosynthesis by up to one order of magnitude. On annual average, carbonate and silicate sands cover ~ 8 % and microbial mat communities ~ 13 % of their photo-metabolic N demand *via* N_2 fixation.

* This chapter is under review at Marine Ecology Progress Series:
Bednarz VN, van Hoytema N, Cardini U, Naumann MS, Al-Rshaidat MMD, Wild C

Introduction

Coral reefs are characterized by high benthic community biomass and primary production despite being surrounded by oligotrophic waters (e.g. Odum & Odum 1955, Gattuso et al. 1998). Up to 90 % of total carbon (C) fixation on coral reefs is derived from benthic photosynthetic primary production, where the highest production is often associated with corals (zooxanthellae), turf algae or macroalgae (Kinsey 1985, Gattuso et al. 1998). In comparison, the sand-associated microphytobenthos displays lower primary productivity rates per unit surface area, but given the often large areal extent of unconsolidated sandy sediments in reefs, net microphytobenthic primary productivity may be on the same order of magnitude and equally important as coral or macroalgal production (Kinsey 1985, Clavier & Garrigue 1999, Werner et al. 2006, Garren & Azam 2012).

Besides primary productivity reef sands represent an important biocatalytical filter system for organic matter (Wild et al. 2004a, Wild et al. 2004b, Werner et al. 2006). The relatively large grain size of reef sands ensures high permeability (> 10^{-12} m^2) for water exchange and provides settling space for microphytobenthic communities, which both represent key factors for efficient organic matter degradation and concomitant nutrient recycling (Rasheed et al. 2003a, Wild et al. 2004a, Wild et al. 2004b, Werner et al. 2006). Reef sands generally contain 10^3-times more bacteria and up to 80-times higher nutrient concentrations than the surrounding seawater (Rasheed et al. 2002) highlighting the importance of this reef compartment for nutrient recycling in oligotrophic reef environments (Garren & Azam 2012). As oligotrophic reefs receive low amounts of allochthonous nutrient input, they strongly rely on the efficient recycling and new generation of nutrients (Howarth 1988). In particular, nitrogen (N) is mostly the limiting nutrient for primary productivity in coral reefs (Eyre et al. 2008).

Besides recycling of essential nutrients, measurements of dinitrogen (N_2) fixation indicate that reef sands also play an important role for the generation of new bioavailable N (Shashar et al. 1994, Charpy-Roubaud et al. 2001). Capone et al. (1992) found that N_2 fixation in the top layers (0 – 2 cm) of reef sediments accounted for more than 50 % of the total sedimentary ammonium production. Biological N_2 fixation is a physiological process unique to diazotrophic prokaryotes and, yet being energy-costly, can represent an alternative nutrient supply if growing under N-limited ambient conditions typical for coral reef environments (Charpy-Roubaud et al. 2001, Scanlan & Post 2008). In coral reefs several benthic substrates (e.g. sand, coral rubble, cyanobacterial mats and living corals) are actively fixing N_2 (Cardini et al. 2014). Since reef sands can cover large areas on a reef, previous studies have highlighted the magnitude of sedimentary N_2 fixation and its importance for the N requirement of the total reef benthos (Shashar et al. 1994, Casareto et al. 2008, Charpy et

al. 2001, 2010). Shashar et al. (1994) calculated for a lagoon in the Northern Red Sea that reef sands contribute ~70 % to the total N_2 fixation within the reef, while Charpy-Roubaud et al. (2001) estimated that sedimentary N_2 fixation covers ~24 % of the annual N requirements for the total benthic primary productivity in the Tikehau Lagoon (French Polynesia).

The dominant sand type in reef environments is biogenic carbonate sand, while in some regions terrigenous silicate sands co-occur. At the Northern Red Sea, the rare occurrence of flood events through otherwise desiccated river mouths lead to the deposition of silicate sands in many fringing reefs of the area. These two sand types are exposed to identical, seasonally variable environmental conditions but exhibit different physico-chemical characteristics in grain size, surface structure and area, permeability and transparency to light (Table 2.1). Together these factors define two different habitats, which select for sand-specific microbial communities (Schöttner et al. 2011) also affecting sedimentary primary productivity and N_2 fixation rates. Previous studies have demonstrated the importance of microphytobenthic photosynthesis and N_2 fixation for total benthic primary productivity and biogeochemical nutrient cycles within the reef ecosystem (Charpy-Roubaud et al. 2001, Werner et al. 2008). Nonetheless, to our best knowledge, no study has investigated both processes with particularly focus on the effect of sand mineralogy and environmental key parameters (e.g. temperature, light intensity, nutrient concentrations).

Therefore, the main objectives of the present study were 1) to quantify N_2 fixation and microphytobenthic photosynthesis of three different reef sand communities (bare carbonate sands, silicate sands and microbial mats on carbonate sands) in a seasonal resolution in order to investigate the effects of sand type along with seasonally changing environmental key parameters, and 2) to calculate the respective contribution of fixed N to the N requirements for microphytobenthic primary productivity.

Methods

Study site

This study was conducted at the Marine Science Station (MSS) Aqaba at the Northern Gulf of Aqaba, Jordan (29° 27`N, 34° 58`E). The MSS is situated approximately 10 km south of Aqaba City with access to a Red Sea fringing coral reef inside a marine reserve. Strong regional seasonality is reflected by substantial variability of environmental key parameters throughout the year due to the annual water column stratification cycle in the Gulf of Aqaba (Silverman et al. 2007, Carlson et

al. 2014). The hard coral dominated (38.6 ± 2.6 %) fringing reef site reveals an average bare carbonate sand cover of 18.5 ± 2.8 % with highest coverage in 5 m water depth (50.7 ± 6.3 %) followed by the reef flat (19.0 ± 3.9 %) and 10 m water depth (16.2 ± 1.4 %). In 1 m or 20 m depth bare carbonate sand covers less than 4.0 %. Overall, less than 1 % of the total bare carbonate sand area is covered by microbial mat communities throughout the year. Highest microbial mat abundance (~ 3 %) appeared in 5 m water depth with a seasonal development ranging from < 1 % in winter and summer to 5 % in fall and 7 % in spring. The fringing reef is interrupted by a ~ 100 m long area completely covered by silicate sand from the shore down to at least 40 m. This area is almost free of hard coral structures but to 10 to 20 % covered by seagrass beds. In order to study the effect of seasonality on N_2 fixation and primary productivity by microbial communities of the different reef sands, all experiments described below were conducted once in each of the following months representing a respective season: February (winter), April (spring), September (summer), and November (autumn) during the year 2013. Thermal stratification in the Gulf of Aqaba develops from May to November with a maximum during August/September while from January to April deep-water mixing exists reaching the maximum mixing depth in March/April (Manasrah et al. 2006).

Monitoring of environmental parameters

In situ water temperature and light intensity were continuously recorded at the sampling locations (10 m water depth) using data loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: ± 0.53 °C, spectral detection range: 150 – 1200 nm; Bourne, MA, USA). Additional monthly light intensity measurements were performed with a quantum sensor (LI-COR LI-192SA, Lincoln, Nebraska, USA) in order to convert data logger light readings (lux) to photosynthetically active radiation (PAR, µmol quanta m^{-2} s^{-1}, wavelength 400 to 700 nm) using a conversion of 1 µmol quanta m^{-2} s^{-1} = 52 lux. The presented light data are seasonal means of maximum light intensities measured during 11:00 and 13:00 (Table 2.2). Weekly seawater samples were collected from 10 m water depth (approximately 1 m above the sand) using high-density polyethylene canisters (5 L, n = 4) and transported back to the laboratory within 30 min. There, subsamples for inorganic nutrients, chlorophyll *a* (Chl *a*), particulate organic carbon (POC) and particulate nitrogen (PN) were collected. Inorganic nutrient subsamples (50 ml) were filtered through cellulose acetate membrane filters (nominal pore size: 0.45 µm) for determination of dissolved inorganic nitrogen (DIN: ammonium, nitrate and nitrite) and dissolved inorganic phosphate (DIP) following standard methods (Holmes et al. 1999, Strickland & Parsons 1972 and Murphy & Riley 1962). Ammonium

was determined fluorometrically using a Trilogy Fluorometer (Turner Designs), while all other nutrients were measured photometrically with a JASCO-V630 spectrophotometer (Jasco Analytical Instruments). Detection limits for ammonium, DIP, and nitrogen oxides (nitrate and nitrite) were 0.09, 0.01, 0.02 μM, respectively. Chl *a* subsamples (1 L) were filtered onto pre-combusted GF/F filters (nominal pore size: 0.7 μm) and stored frozen at -80 °C in the dark until analysis. Chl *a* was extracted with 90 % acetone (12 h in the dark at 4 °C) and measured using a Trilogy Fluorometer fitted with a non-acidification module (CHL NA #046, Turner Designs). Additional subsamples for POC (1 L) and PN (2 L) were filtered onto pre-combusted GF/F filters, dried in the oven (40 °C, 48 h) and stored dry pending analysis. Prior to analysis dried filters were wrapped in silver foil and POC filters were acidified with 0.1 N HCl to remove any inorganic carbon. POC and PN filter contents were measured on a EuroVector elemental analyser (EURO EA 3000) with analytical precision of ≤0.1% (C) and ≤0.03% (N).

Table 2.1. Sediment properties of carbonate and silicate sand in the Gulf of Aqaba previously measured at the study site (OC: organic carbon, DIN: dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate).

Parameter	Carbonate sand	Silicate sand	Reference
$CaCO_3$ content (%)	75-85	4-6	Rasheed et al. 2003b
Grain size (μm)	559	229	Rasheed et al. 2003b
Permeability (m^{-2} x 10^{-12})	116 ± 11	27 ± 3	Wild et al. 2005
Porosity (%)	47	33	Rasheed et al. 2003b
OC content (%)	0.36	0.24	Rasheed et al. 2003b
OC decomposition (mg m^{-2} d^{-1})	3.0	2.0	Rasheed et al. 2003a
DIN content (μmol L^{-1})	17-20	6-7	Rasheed et al. 2003b
DIP content (μmol L^{-1})	1.4-1.9	0.5-0.6	Rasheed et al. 2003b
Ammonium efflux (mmol m^{-2} d^{-1})	3.41 ± 0.32	2.15 ± 0.26	Rasheed et al. 2003a
DIP efflux (mmol m^{-2} d^{-1})	0.03 ± 0.002	0.02 ± 0.001	Rasheed et al. 2003a
Chl *a* (μg g^{-1})	0.72 ± 0.16	0.63 ± 0.12	Rasheed et al. 2003b
Bacterial cell number (cm^{-3})	3.1 ± 0.9 x 10^9	1.5 ± 0.5 x 10^9	Schöttner et a. 2011

Table 2.2. Summary of key environmental water parameters monitored at 10 m water depth during four seasons (DIN: dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate, POM (POC+PN): particulate organic matter, POC: particulate organic carbon, PN: particulate nitrogen). Values are represented as means (n = 4) with SE in parentheses.

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

Substrate sampling

Two neighbouring back reef sites at 10 m water depth in front of the MSS covered by either carbonate or silicate sand were chosen for substrate sampling using SCUBA. The lateral distance between the two sites was approximately 150 to 200 m, and both sites were in close vicinity (5 m distance) to the adjacent coral reef framework. Both sand types revealed distinct mineralogical, physical and biological characteristics as shown in Table 2.1. Once during each season, carbonate sand (n = 8) and silicate sand (n = 8) samples were taken using custom-made PVC sediment corer (inner diameter: 4.3 cm). Additional carbonate sand samples (n = 8) showing dark-brown microbial mats (~1-2 mm thick) on top were collected within 100 m distance from the bare carbonate sand sampling site. Cores were immediately transported back to the MSS where the top 1 cm surface layer of each core was individually transferred into a petri-dish of equal diameter (planar surface = 14.52 cm^2) before placed into individual incubation glass chambers (500 ml chamber for carbonate and silicate sands, 1000 ml chamber for microbial mats). During all handling, special care was taken to keep the sediment stratification and minimize the exposure time to air (< 30 s). All incubation chambers were kept in an outdoor 800 L flow-through tank under *in*

situ conditions (see below) during subsequent measurements of sedimentary oxygen (O_2) fluxes and N_2 fixation over the next two days.

Quantification of O_2 fluxes

All following incubations took place in the outdoor 800 L flow-through aquarium supplied with seawater pumped directly from the reef at the 10 m sampling depth (exchange rate: 4000 L h^{-1}) to ensure *in situ* water temperature and nutrient concentrations. Light intensity was monitored with lux and PAR data loggers (see above) and adjusted with black netting to those measured *in situ* at 10 m water depth. O_2 fluxes of the sand samples as a proxy for primary productivity was quantified in two individual incubations. The first incubation was carried out on the sample collection day 1-2 h after sunset to measure dark respiration (R), while the second incubation was started the following day at 12:00 for net photosynthesis (P_{net}) determination. Each sand substrate (n = 8) was incubated individually and additional chambers (500 ml, n = 8) only filled with seawater served as controls to measure planktonic background metabolism. Chambers were sealed and incubated under constant stirring (600 rpm) for 2-6 h (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific™). O_2 concentrations were measured at the beginning and end of each incubation period using a salinity- and temperature-corrected O_2 optode sensor (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). To calculate O_2 fluxes during P_{net} and R incubations, O_2 start concentrations were subtracted from end concentrations, and the results were normalized by incubation time. Finally, O_2 fluxes were corrected for the seawater control signal related to the chamber volume and normalized to the sand surface area (nmol O_2 cm^{-2} h^{-1}). Gross photosynthesis (P_{gross}) rates were calculated according to $P_{gross} = P_{net} - R$. In order to calculate the N requirement for P_{gross} the daily O_2 production was calculated assuming a daily 12 h photoperiod and values were converted into C fluxes using a community photosynthetic (PQ) and respiratory quotient (RQ) of 1.0 (1 mol O_2 = 1 mol C) according to Taddei et al. (2008) who experimentally determined similar PQ and RQ values for coral reef sands.

Quantification of N_2 fixation

N_2 fixation rates were quantified 3 - 4 h after the P_{net} incubation ended applying a modified acetylene (C_2H_2) reduction technique (Capone 1993, Wilson et al. 2012). C_2H_2 gas was freshly generated from calcium carbide and bubbled through fresh seawater in order to produce C_2H_2-

enriched seawater. Incubations were conducted in 500 ml glass chambers containing 400 ml natural seawater of which 10 % were replaced with C_2H_2-enriched seawater. Chambers were immediately sealed gas-tight with a spring-loaded glass lid equipped with a rubber injection port on top for gas sampling and 10 % of the air headspace was replaced by freshly generated C_2H_2 gas. In addition, 4 different sets of controls were tested for the reduction of C_2H_2 to ethylene (C_2H_4) production: 1. unfiltered seawater control (without sand samples, n = 8); 2. 0.2 µm-filtered seawater control (without sand samples, n = 6); 3. petri-dish (without sand sample) in unfiltered seawater (n = 6); 4. sand sample in unfiltered seawater without C_2H_2 addition (natural C_2H_4 production, n = 6). Over the entire incubation period (24 h), all chambers were magnetically stirred as described above and gas samples were taken at 0, 4, 12, 16 and 24 h. At each of these time intervals, 1 ml of gas sample was collected with a gastight syringe from each chamber, transferred into gastight 2 ml vials previously filled with distilled water, and stored frozen upside down until analysis. C_2H_4 concentrations of gas samples were measured in the field laboratory using a reducing compound photometer (RCP) (Peak Laboratories) with a detection limit of 100 ppb. Calibration of the RCP was conducted using serial dilutions of a 200 ± 4 ppm C_2H_4 standard in air (Restek, Bellefonte, PA, USA).

The C_2H_4 evolution in each incubation chamber was calculated according to Breitbarth et al. (2004). Values were finally corrected for the unfiltered seawater control signal related to the chamber volume and normalized to incubation time and sand planar surface area. All rates are reported as means ± SE and in C_2H_4 production rates (nmol C_2H_4 cm^{-2} h^{-1}) to allow good comparison to previous studies using the C_2H_2 reduction assay. C_2H_4 rates were only converted to N_2 fixation rates in order to calculate the percentage contribution by N_2 fixation to the N requirements for microphytobenthic primary production. Since no parallel ^{15}N calibration was applied a theoretical ratio of 3 mol C_2H_2 reduced to 1 mol N_2 fixed was used which has been previously found for white coral reef sands dominated by diatoms and dinoflagellates (Charpy-Roubaud et al. 2001).

Statistical analysis

All statistical analyses were carried out using Primer-E version 6 software (Clarke & Gorley 2006) with the PERMANOVA+ add on (Anderson 2001). Analyses were based on Bray Curtis similarities of the physiological parameters (square root transformed). Two-factor PERMANOVAs were performed to test for differences of the parameters N_2 fixation, P_{gross} and R rates between

substrate type and season. Therefore, type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations), and pairwise-tests were carried out if significant differences occurred. Finally, correlations between N_2 fixation rates and the environmental water parameters as well as between N_2 fixation and sedimentary O_2 fluxes (P_{gross} and R) were determined *via* linear regression.

Results

Environmental key parameters

All monitored environmental key parameters exhibited a strong seasonal patterns (Table 2.2) with the most distinct differences between the stratified (summer and fall) and deep-water mixed (winter and spring) season. Highest irradiance (PAR) was measured in spring and summer compared to winter and fall (Table 2.2). Summer also revealed the highest water temperature before it starts decreasing during fall until annual minimum values during winter and spring are reached. Inorganic nutrients (DIN and DIP) were negatively correlated to water temperature with at least twice as high concentrations during winter and spring compared to summer and fall thereby clearly reflecting the seasonal change between stratification and deep-water mixing of the water column. The calculated DIN:DIP ratio ranged from 5.31 to 11.25 throughout the year but was consistently lower than the Redfield ratio (16:1) indicating N limited conditions in the water column, particularly during summer. N limitation is further suggested by the ratio of POC:PN in the water column that always exceeded the Redfield ratio (106:16). POC and PN revealed highest concentrations during spring together with highest chl *a* concentrations in the water thereby indicating the seasonal plankton bloom and the increased production of biomass during this period of the year.

O_2 fluxes by reef sand communities

P_{gross} rates averaged 270 ± 25 nmol O_2 cm^{-2} h^{-1} for carbonate sand and 233 ± 17 nmol O_2 cm^{-2} h^{-1} for silicate sand across all seasons. Both bare sands exhibited similar P_{gross} rates during each season except during spring when carbonate sand exhibited significantly higher rates compared to silicate sand. The seasonal pattern was similar with significantly increased P_{gross} rates during spring and summer for both sands (Fig. 2.1, Table 2.3). Microbial mats showed no seasonal variation of

P_{gross} rates but the annual average of 809 ± 43 nmol O_2 cm^{-2} h^{-1} was three times higher compared to carbonate and silicate sand. R was on annual average almost twice as low in carbonate (-70 ± 3 nmol O_2 cm^{-2} h^{-1}) compared to silicate sand (-126 ± 12 nmol O_2 cm^{-2} h^{-1}). While carbonate showed significantly higher R rates during spring and summer, R in silicate sand peaked during winter and summer. R rates of microbial mats was significantly highest during summer and averaged -135 ± 7 nmol O_2 cm^{-2} h^{-1} over all seasons, thus being in the range of R measured for silicate sands.

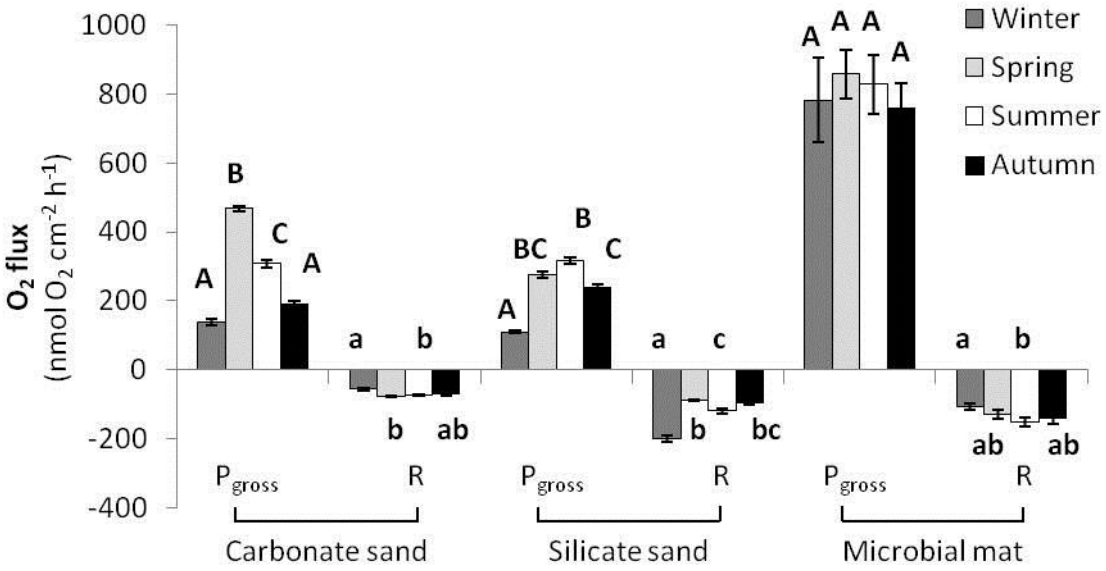

Figure 2.1. P_{gross} and R rates measured as O_2 fluxes in the substrates carbonate sand, silicate sand and microbial mat during four seasons (winter, spring, summer, autumn). Values are given as mean (n = 8) ± SE. Different letters indicate significant differences for P_{gross} (A-C) and R (a-c) rates between the four seasons for each substrate type, respectively, based on pair-wise PERMANOVA analysis.

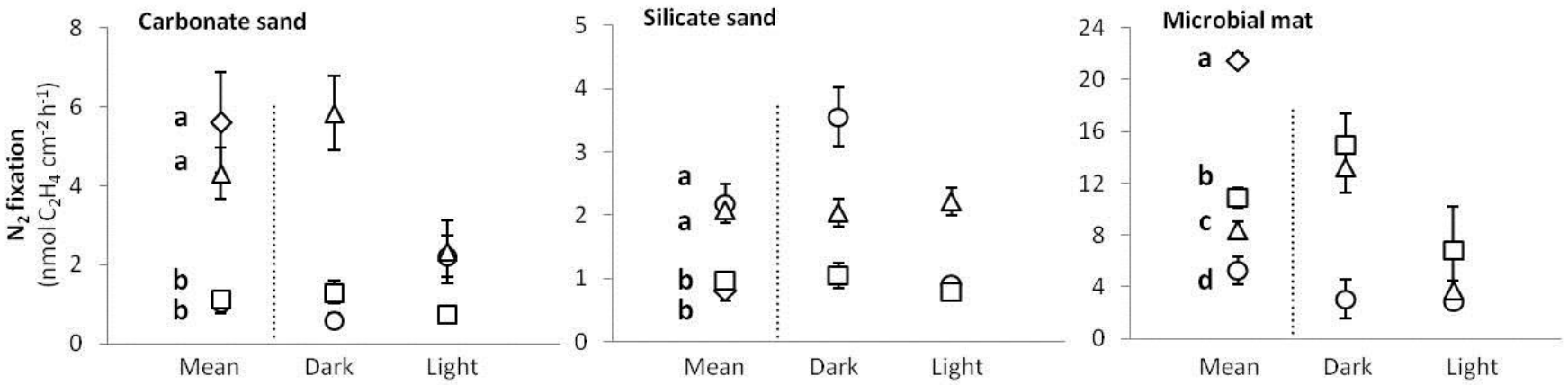

Figure 2.2. Mean N_2 fixation (C_2H_4 production) rates of the different substrates (carbonate sand, silicate sand, microbial mat) measured during winter (circles), spring (diamonds), summer (triangles) and autumn (quadrates) over a 24 h incubation period. For the winter, summer and autumn incubations, N_2 fixation rates for the dark and light periods are separately presented. Values are given as mean (n = 8) ± SE. Different letters (a-d) indicate significant differences between the four seasons for each substrate type, respectively, based on pair-wise PERMANOVA analysis.

N_2 fixation by reef sand communities

On annual average, N_2 fixation by carbonate sand communities (2.88 ± 0.41 nmol C_2H_4 cm^{-2} h^{-1}) was significantly higher when compared to silicate sand (1.52 ± 0.15 nmol C_2H_4 cm^{-2} h^{-1}). The two sands revealed a specific seasonal variability in N_2 fixation rates (Fig. 2.2, Table 2.3). Carbonate sand was significantly more active during spring and summer thereby following the seasonal pattern of P_{gross}. This is supported by a significant positive linear relationship with N_2 fixation explaining 69 % of the variation in P_{gross} (Table 2.4). In contrast, silicate sand revealed significantly highest N_2 fixation activity during winter and summer similar to seasonal maxima of R rates. Correlation revealed a significant positive linear relationship between the two processes with 38 % of the variation in R being explained by N_2 fixation (Table 2.4). Overall, seasonal N_2 fixation variability was more pronounced in carbonate (1.14 to 5.25 nmol C_2H_4 cm^{-2} h^{-1}) compared to silicate sand (0.81 to 2.42 nmol C_2H_4 cm^{-2} h^{-1}). Correlations to the key environmental parameters revealed for N_2 fixation of carbonate sand a significant positive linear relationship to light intensity and POM content in the water, while N_2 fixation of silicate sand was negatively correlated to POM content but not to light intensity (Table 2.4). Additionally, N_2 fixation of both sands showed a significant negative relationship to the DIN:DIP ratio in the water column.

Compared to the two bare reef sands, N_2 fixation activity associated with microbial mats was always almost one order of magnitude higher, seasonal average: 11.95 ± 1.16 nmol C_2H_4 cm^{-2} h^{-1}. N_2 fixation in microbial mats was significantly different between each season with highest rates in spring, followed by fall, summer and winter (Table 2.3). However, no significant relationship was found between N_2 fixation activity and the key environmental water parameters (Table 2.4). Correlation analysis between N_2 fixation and O_2 fluxes in microbial mats revealed significant positive relationships to both P_{gross} and R (Table 2.4).

Besides the seasonal variability of N_2 fixation averaged over 24 h, all three substrates revealed specific dark and light N_2 fixation rates with either similar dark and light N_2 fixation or relatively higher dark N_2 fixation on a 24 h basis (Fig. 2.2). Higher dark than light N_2 fixation was measured for carbonate sand during summer, for silicate sand during winter and for microbial mat communities during summer and autumn.

Table 2.3. Results of two-factorial PERMANOVAs for N_2 fixation, P_{gross} and R rates for the substrate types (carbonate sand, silicate sand and microbial mat) during the four investigated seasons (winter, spring, summer and autumn) in 2013. Substrate and season were fixed effects. PERMANOVA was based on Bray Curtis similarity after square root transformation. Type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations). Significant *p* values are in bold.

Variables	Effect	*df*	SS	MS	Pseudo *F*	*p* value
N_2 fixation	Substrate (Su)	2	31607	15804	140.95	**0.001**
(nmol C_2H_4 cm^{-2} h^{-1})	Season (Se)	3	5384	1795	16.01	**0.001**
	Su x Se	6	11707	1951	17.40	**0.001**
	Residuals	76	8521	112		
	Total	87	57219			
P_{gross}	Substrate (Su)	2	15933	7967	150.12	**0.001**
(nmol O_2 cm^{-2} h^{-1})	Season (Se)	3	3990	1330	25.06	**0.001**
	Su x Se	6	2264	377	7.11	**0.001**
	Residuals	76	4033	53		
	Total	87	26221			
R	Substrate (Su)	2	4204	2102	44.89	**0.001**
(nmol O_2 cm^{-2} h^{-1})	Season (Se)	3	358	119	2.55	0.052
	Su x Se	6	2385	398	8.49	**0.001**
	Residuals	76	3559	47		
	Total	87	10507			

Discussion

Primary productivity and N_2 fixation by reef sand communities

This is the first study comparatively describing primary productivity and N_2 fixation activity of carbonate and silicate reef sand communities. We investigated the top sediment layer where highest diazotrophic activity occurs (Werner et al. 2008). The top sediment layer of both sands can be characterized as net-autotrophic and largely independent from allochthonous C input as P_{gross} rates largely exceeded R rates. N_2 fixation rates for carbonate and silicate sands presented here agree well with values previously measured at different reef locations (Table 2.4). Shashar et al. (1994) measured higher, yet variable N_2 fixation rates in reef sediments from a close site in the Gulf of Aqaba (Eilat, Israel). These differences may be explained by the use of mixed grain sizes ranging from gravel (5 mm) to fine (0.1 mm) and a higher proportion of large grain sized sands, while the present study measured N_2 fixation exclusively in fine grained sands (0.2 to 0.6 mm; Table 2.1).

The present study measured significantly higher N_2 fixation rates in carbonate sand than in silicate sand, and this may be explained by sediment type-specific characteristics. The larger grain size and porosity of carbonate sand increases both permeability and specific surface area (Rasheed et al. 2003a, Wild et al. 2005). Increased permeability generates advective driven fluid fluxes between the sediment and the overlying water thereby enhancing solute exchange and flux of suspended organic matter (Rasheed et al. 2003a), while increased surface area increases the available substrate for microbial community growth. These characteristics support microbial abundance in carbonate sands that largely exceeds cell numbers in silicate sands (Schöttner et al. 2011, Wild et al. 2004a, 2006). Furthermore, significantly higher organic matter degradation and C turnover rates in carbonate sand are caused (Rasheed et al. 2003a, Wild et al. 2005) which increase organic substrate availability (Table 2.1, Rasheed et al. 2003b). This has previously been described as a main factor controlling N_2 fixation activity in shallow carbonate sediments (O'Neil & Capone 1989). Since N_2 fixation represents an energetically costly process (due to breakage of the N_2 triple-bond), diazotrophs have a high need for energy-rich organic substrates, and thus may benefit from the higher organic C content in carbonate compared to silicate sands (Table 2.1). Furthermore, Schöttner et al. (2011) investigated microbial communities of carbonate and silicate sand in the same area and identified sand type as main factor structuring sediment-associated microbial assemblages. Similarly, diazotrophic assemblages likely differ between the two sands. Overall, the present findings highlight the influential role of sediment-specific characteristics (e.g. grain size, permeability, diazotrophic composition) in controlling sediment-associated N_2 fixation activities.

Unconsolidated reef sands also provide open space for the development of microbial mats which are often dominated by cyanobacteria communities and represent important contributors to benthic primary productivity and N supply on coral reefs (Charpy et al. 2010, 2012, Cardini et al. 2014). The presented values for N_2 fixation compare well with values previously reported for benthic microbial mats in other coral reef ecosystems (Table 2.4). Compared to bare carbonate sand, N_2 fixation and P_{gross} rates of microbial mats were ~4.5 and ~3 times higher, respectively, thus indicating a higher *de novo* input of N relative to photosynthetically fixed C. This increased N availability may enable rapid accumulation of biomass and the formation of dense mats in an extremely oligotrophic environment. This is supported by the present study displaying highest microbial mat development and abundance during spring, the season also showing highest year-round N_2 fixation activity by the mats. Nevertheless, all microbial mats in the study site were of small size and overall covered < 1 % of the bare carbonate sand area on the reef. Considering such low coverage by microbial mats compared to bare carbonate sand (18 % of total reef area), the

contribution of bare reef sand areas to total benthic N_2 fixation is likely much higher despite the lower fixation rates per unit of surface area.

Table 2.4. Linear regression analysis between N_2 fixation rates of the three sand substrates (carbonate sand, silicate sand, microbial mat) and both the key environmental water parameters (DIN: dissolved inorganic nitrogen, DIP: dissolved inorganic phosphate, POM: particulate organic matter) and the O_2 fluxes (P_{gross}: gross photosynthesis, R: dark respiration) of the sand substrates. Data presented as R^2 values at significant levels of * < 0.05, ** < 0.005, *** < 0.001. Bold characters indicate a significant positive relationship and italicized characters indicate a significant negative relationship.

Parameter	Carbonate sand	Silicate sand	Microbial mat
Environmental factor			
Irradiance	**0.491*** **	0.023	0.057
Temperature	0.045	0.048	0.052
DIN	0.017	0.009	0.043
DIP	0.003	0.002	0.033
DIN:DIP	*0.189**	*0.259***	0.048
POM	**0.212***	*0.467****	0.006
Sedimentary O_2 fluxes			
P_{gross}	**0.690*** **	*0.153**	**0.568*** **
R	**0.215***	**0.375*** **	**0.610*** **

Seasonal variability of primary productivity and N_2 fixation

This is the first study investigating the response of sediment-associated primary productivity and N_2 fixation to seasonal changing environmental conditions. Carbonate and silicate sands were exposed to similar changing environmental conditions, thus differences in the biological variables reflect a sand type specific response. Overall, seasonal variability was more pronounced in carbonate than in silicate sands. This is most likely due to sand-specific differences in permeability, specific surface area, microbial community and mineralogy leading to tighter benthic-pelagic coupling between the water column and sediment pore-water in carbonate sand. Therefore,

seasonal variation in water column nutrient availability will more directly affect the nutrient inventory in the upper sediment layer (0 to 2 cm) of carbonate than silicate sand (Rasheed et al. 2003b). Schöttner et al. (2011) investigated the effects of season, sediment depth and location on microbial community structure in reef sediments in the Gulf of Aqaba and found that season was the most significant structuring factor in carbonate sands, while sediment depth was more influential in silicate sands. Seasonality and sediment depth may also determine the diazotrophic community structure, thus explaining the stronger seasonal variation in N_2 fixation activity observed for carbonate compared to silicate sand in the present study.

N_2 fixation in carbonate sand was primarily stimulated during spring and summer by seasonally increased ambient light and POM availability. This agrees with previous studies describing light as a main factor influencing sedimentary N_2 fixation (Charpy-Roubaud et al. 2001, Charpy et al. 2007, Werner et al. 2008) and suggests the dominance of phototrophic diazotrophs. The increased N_2 fixation rates in carbonate sand are mainly due to elevated diazotrophic activity during night, indicating a shift towards a more non-heterocystous bacterial community. Non-heterocystous diazotrophs separate the O_2-sensitive N_2 fixing nitrogenase enzyme complex temporally from O_2 producing photosynthesis, whereas heterocystous diazotrophs can fix N_2 also during daylight in specialized O_2-free cells (heterocyst). Night-time N_2 fixation activity also depends on photosynthetic energy supply and correlates positively to the intensity of the previous daylight period (Charpy et al. 2007). Furthermore, N_2 fixation activity heterotrophically profits from available organic C sources. Thus, the two-fold higher POM supply *via* sedimentation during spring and summer (Wild et al. 2009) likely provides additional energy for sediment associated N_2 fixation. Despite seasonal changes in POM availability, carbonate sand communities revealed little seasonal variation in R rates, while primary productivity responded similar as N_2 fixation to seasonality. This is in line with previous studies (Rasheed et al. 2002, 2003b, Wild et al. 2009) and suggests that the microphytobenthos is largely independent from allochthonous C input and likely sustains its primary productivity *via* N_2 fixation.

N_2 fixation in silicate sand was negatively correlated to P_{gross}, positively to R and was not influenced by ambient light availability, thus it strongly indicates the dominance of heterotrophic diazotrophs. Although activity of heterotrophic diazotrophs completely relies on external organic C sources, N_2 fixation in silicate sand was negatively related to POM concentrations in the water column. This implies a minor organic C supply and trophic link between the sediment and the overlaying water and is further supported by a more slowly transport of organic substrates through the rather diffusion-limited silicate sands compared to the highly advection-driven carbonate sands

(Rasheed et al. 2003b). Despite a sand-specific seasonal response, N_2 fixation of both sands negatively correlate to the DIN:DIP water column ratio. The low DIN:DIP ratio over the year indicates N limited conditions and suggest N_2 fixation as an advantageous strategy for sedimentary primary productivity.

Contribution of N_2 fixation to primary productivity

The significant linear correlation between N_2 fixation and P_{gross} suggests a tight coupling between the two processes. Averaged over all seasons, daily P_{gross} in carbonate sand, silicate sand and microbial mats was calculated to require 4.89, 4.21 and 14.66 mmol N m^{-2} d^{-1}, respectively assuming the Redfield ratio (106:16) for primary productivity applicable to microphytobenthic communities of reef sands (Delesalle et al. 1998, Charpy-Roubaud et al. 2001, Werner et al. 2008). Thus, on annual average, N_2 fixation rates measured here would supply 8.4, 8.1 and 13.3% of the total N needed for microphytobenthic primary productivity in carbonate sand, silicate sand and microbial mats, respectively (Table 2.6). These estimates are similar to a New Caledonian reef lagoon, where N_2 fixation in reef sands and microbial mat communities contributed between 5% and 21% of the N required for primary productivity (Charpy et al. 2007, 2010). Also at Sesoko, Japan similar contributions of 5.7% for sandy bottoms and 10.0 to 26.5% for microbial mats were calculated (Casareto et al. 2008). However, these estimates likely underestimate the contribution of N_2 fixation as a substantial quantity of N is recycled (autochthonous N-input) within the reef sediments (Crossland et al. 1991, Charpy-Roubaud et al. 2001) thereby largely reducing the photometabolic demand for 'new' N (allochthonous N-input) but increasing the relative N input *via* N_2 fixation.

DIN fluxes from the sediment to the overlaying waters were shown to importantly fuel primary productivity of the whole reef benthos (Charpy-Roubaud et al. 1996, 2001, Rasheed et al. 2002). In the study site, carbonate sand shows a 2.8 higher DIN content and 1.6 higher ammonium efflux to the overlaying water compared to silicate sand, which may be explained by its generally higher N_2 fixation and organic matter degradation rates (Table 2.1, Rasheed et al. 2003a, 2003b). Overall, the present findings highlight the significant role of N_2 fixation as important N source for sedimentary primary productivity and by releasing large quantities of fixed N to the overlaying water reef sediments, particularly carbonate sands, may importantly support primary productivity of other benthic organisms and of the entire coral reef ecosystem.

Table 2.5. Acetylene reduction (AR; nmol C_2H_4 cm^{-2} h^{-1}) and inferred N_2 fixation rates (NF; mmol N m^{-2} d^{-1}) of the different reef sand communities investigated in the present study in comparison with values reported from other coral reef areas worldwide (GBR: Great Barrier Reef). AR:NF is the respective C_2H_2:N_2 conversion ratio used to calculate NF from AR.

Substrate	AR	AR:NF	NF	Location	Method	Reference
Carbonate sands	2.88 ± 0.41	3	0.46 ± 0.07	Red Sea	C_2H_2	Present study
	0.04-2.32	4	0.01-0.28	Caribbean	C_2H_2	O'Neil & Capone 1989
	0.75-1.95	3	0.12-0.31	GBR, Australia	C_2H_2	Capone et al. 1992
	19.52 ± 17.50	4	2.34 ± 2.10	Red Sea	C_2H_2	Shashar et al. 1994
	0.18-1.02	1.8-4.8*	0.03-0.28	French Polynesia	C_2H_2; $^{15}N_2$	Charpy-Roubaud et al. 2001
	-	-	0.10-0.16	Ishigaki Island	$^{15}N_2$	Miyajima et al. 2001
	0.32	1.6*	0.34	French Polynesia	C_2H_2; $^{15}N_2$	Charpy-Roubaud & Larkum 2005
	9.76 ± 3.21	4	1.17 ± 0.39	New Caledonia	C_2H_2	Charpy et al. 2007
	0.03-0.12	3	0.004-0.019	GBR, Australia	C_2H_2	Werner et al. 2008
Silicate sands	1.52 ± 0.15	3	0.24 ± 0.02	Red Sea	C_2H_2	Present study
Microbial mats	11.95 ± 1.16	3	1.91 ± 0.19	Red Sea	C_2H_2	Present study
	2.7-47.8	4	0.3-5.7	California	C_2H_2	Paerl et al. 1993
	0.96	1.6*	0.57	French Polynesia	C_2H_2; $^{15}N_2$	Charpy-Roubaud & Larkum 2005
	0.59-2.97	4	0.07-0.36	Indian Ocean	C_2H_2	Charpy et al. 2012

* Conversion factor was empirically determined

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Chapter III

Seasonality in dinitrogen fixation and primary productivity by coral reef framework substrates from the northern Red Sea*

Abstract

High rates of N_2 fixation by coral reef benthic substrates may play an important role in supporting reef gross photosynthesis (P_{gross}). However, little is known regarding the influence of environmental parameters on coral reef benthic N_2 fixation. This study quantified N_2 fixation (by acetylene reduction) and P_{gross} by three abundant reef framework substrates: turf algae, coral rock, and the abundant encrusting sponge *Mycale fistulifera*, over four seasons in the northern Gulf of Aqaba. N_2 fixation activity was detected during day and night for all substrates but on annual average was significantly higher for turf algae (4.4 ± 3.9 nmol C_2H_4 cm^{-2} h^{-1}) and coral rock (3.5 ± 2.8 nmol C_2H_4 cm^{-2} h^{-1}) compared to *M. fistulifera* (0.2 ± 0.2 nmol C_2H_4 cm^{-2} h^{-1}). There was strong seasonal variability in N_2 fixation, with rates for all substrates one order of magnitude higher in summer compared to winter, coinciding with highest irradiance and temperature, but lowest inorganic nutrient concentrations. Increased N_2 fixation in summer corresponded with an increase in P_{gross} by turf algae and *M. fistulifera*, revealing a significant positive relationship between the two processes. N_2 fixation contributed 7 and 9%, respectively, to the N demand of P_{gross} in coral rock and turf algae on annual average, and this contribution increased in summer to 13 and 17%. These findings highlight the role of environmental parameters in regulating benthic substrate-associated N_2 fixation and the importance of fixed N in supporting primary productivity, particularly during the nutrient-depleted summer season in the Gulf of Aqaba.

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Introduction

Coral reefs are characterized by high productivity but typically are surrounded by oligotrophic waters (Odum & Odum 1955, Hatcher 1988), where nitrogen (N) is a key limiting nutrient for growth (Littler et al. 1991, Eyre et al. 2008). Efficient nutrient cycling in the benthos contributes to this high productivity, but input of new N is essential to sustain net ecosystem production and growth. Though energetically costly, numerous studies indicate that dinitrogen (N_2) fixation represents a substantial source of new N on coral reefs (Webb et al. 1975, Larkum et al. 1988, O'Neil & Capone 1989, Charpy et al. 2007).

Biological N_2 fixation is carried out by a diverse group of heterotrophic and photoautotrophic bacteria (Zehr et al. 2003, Kneip et al. 2007), but cyanobacteria are a key contributor to benthic N_2 fixation on coral reefs (Casareto et al. 2008, Charpy et al. 2012). Cyanobacterial mats have attracted much research focus due to their high N_2 fixation rates (e.g. Charpy et al. 2007, Diez et al. 2007, Bauer et al. 2008, Casareto et al. 2008). However, cyanobacteria are also important components of the various reef framework substrates that are ubiquitous on coral reefs; including algal turfs, endolithic algal communities associated with calcium carbonate structures, and endosymbiotic communities of sponges (Charpy et al. 2012). In coral reefs in the northern Gulf of Aqaba, these reef framework substrates are a dominant component of the benthos. Turf algae can make up 72% of the benthic community on reefs in Eilat (Israel), while on the Jordanian side of the Gulf, biogenic reef framework with only sparse epilithic overgrowth (hereafter: coral rock) can account for up to 58% of the benthic cover (Bahartan et al. 2010). High rates of N_2 fixation have been measured in both turf algae and coral rock (eg. Larkum et al. 1988, Williams & Carpenter 1998), therefore these reef framework substrates may contribute importantly to fixed N on reefs in the Gulf of Aqaba. Evidence for active N_2 fixation in sponges is scarce (Wilkinson & Fay, 1979, Shashar et al. 1994a), but many species harbor microbial symbionts capable of fixing N (Taylor et al. 2007, Mohamed et al. 2008) suggesting N_2 fixation in sponges may be widespread.

Due to their association with photosynthetic cyanobacteria and algae, reef framework substrates also contribute to reef primary productivity. Turf algae are dominant primary producers on many reefs (Adey & Goertemiller 1987, Carpenter & Williams, 2007) and more than one third of sponges in the Caribbean, Great Barrier Reef (GBR) and West Indian Ocean harbor photosynthetic symbionts (Wilkinson 1987, Steindler et al. 2002, Erwin & Thacker 2007). Due to the oxygen (O_2) sensitivity of nitrogenase, the enzyme responsible for N_2 fixation, photosynthesizing diazotrophs have evolved strategies to allow photosynthesis and N_2 fixation to

co-occur (Berman-Frank et al. 2003). Spatial separation in heterocystous cyanobacteria allows the fixation of N_2 during the day (Gallon 2001), while non-heterocystous cyanobacteria typically fix N_2 at night, relying on energy derived from the carbon (C) fixed during the previous daylight period (Bergman et al. 1997, Charpy et al. 2007). In marine sponges, hypoxic zones may facilitate O_2-sensitive processes such as N_2 fixation (Hoffman et al. 2005). N_2 fixation supports pelagic primary productivity (Mulholland et al. 2006) and may likewise support photosynthesis in coral reef framework substrates. However, little is known regarding the interaction between N_2 fixation and photosynthesis in benthic substrates, and few studies have quantified both processes in parallel.

Fringing reefs in the Gulf of Aqaba experience strong seasonal variation in key environmental parameters due to the annual stratification cycle in the water column. Winter and early spring are characterized by low temperature and irradiance but high inorganic nutrient concentrations as deep convective mixing of the water column transports nutrient-enriched deep water into the photic zone (Carlson et al. 2014). Increased irradiance followed by warming sea surface temperatures throughout spring and summer lead to the development of a thermocline with a nutrient-depleted surface layer (Silverman et al. 2007), resulting in summer conditions of high temperature and irradiance but low inorganic nutrient concentrations. These environmental parameters are known to influence planktonic N_2 fixation (Sohm et al. 2011), but their effect on benthic diazotrophs is largely unknown (Cardini et al. 2014).

The objectives of this study, therefore, were 1) to quantify N_2 fixation and primary productivity in three dominant reef framework substrates; turf algae, coral rock, and an encrusting sponge in seasonal resolution in order to evaluate the effect of seasonally variable key environmental parameters on these processes, and 2) to determine the contribution of N_2 fixation to the N requirements for primary production in the three investigated substrates.

Methods

Study site

This study was conducted in the northern Gulf of Aqaba at the Marine Science Station (MSS) Aqaba, Jordan (29°27' N, 34°58' E). Sampling was carried out on the 1 km long fringing reef in front of the MSS, which is designated as a marine reserve. All experimental work was carried out in the MSS laboratories. In order to examine the effect of seasonality, all experiments were repeated

over four seasonal periods in 2013: winter (February), spring (April), summer (September), and fall (November).

The benthic reef community was dominated by hard and soft corals, while coral rock represented the third most abundant benthic substrate type at 10 m water depth, covering on average 14.2 ± 5.0% of the available substrate. The percent cover of turf algae (annual average: 4.4 ± 4.5%) was seasonally variable reaching a maximum of 10.3 ± 4.2% in winter and decreasing to a minimum of 1.0 ± 1.0% in fall. Sponge cover was constant throughout the year averaging 1.2 ± 0.9%. The non-cryptic sponge community was dominated by abundant encrusting sponge *Mycale fistulifera*, which accounted for 65% of the visible sponge cover at 10 m water depth. Together the three investigated substrates accounted for 19.8 ± 10.3% of the total benthic coverage.

Environmental monitoring

In situ water temperature and irradiance were continuously monitored at the sampling site (10 m water depth) using data loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: ± 0.53 °C, spectral detection range: 150 – 1200 nm). Parallel irradiance measurements with a quantum sensor (Model LI-192SA; Li-Cor) allowed the conversion of lux measurements to photosynthetically active radiation (PAR µmol photons m^{-2} s^{-1}, wavelength 400 - 700 nm) using a conversion factor of 1 µmol quanta m^{-2} s^{-1} = 52 lux. Irradiance data are presented as seasonal means (± SD) of daily maximum values (Table 3.1). Weekly seawater samples (n = 4) were collected by SCUBA using high-density polyethylene canisters (5 L) at 10 m water depth (~1 m above the bottom) and immediately transferred to the laboratory for further processing. Subsamples (n = 4) were taken for quantification of inorganic nutrients, particulate organic carbon (POC), particulate nitrogen (PN), and chlorophyll *a* (Chl *a*). Inorganic nutrient subsamples were filtered through cellulose acetate filters (nominal pore size 0.45 µm) for determination of ammonium (NH_4^+), nitrate (NO_3^{2-}), nitrite (NO_2^-), and phosphate (PO_4^{3-}) concentrations following standard methods (Murphy & Riley 1962, Strickland & Parsons 1972, Holmes et al. 1999). NH_4^+ was determined fluorometrically using a Trilogy Fluorometer (Turner Designs), while all other nutrients were measured photometrically with a JASCO-V630 spectrophotometer (Jasco Analytical Instruments). The detection limits for NH_4^+, PO_4^{3-}, and NO_x were 0.09, 0.01, and 0.02 µM, respectively. Subsamples for Chl *a* determination (n = 4, 1 L) were filtered onto pre-combusted (450°C, 4 h) GF/F filters (VWR: nominal pore size 0.7 µm) and stored frozen at -80 °C in the dark until further processing. Chl *a* was extracted with 90% acetone (12 h in the dark at 4°C) and

analysed fluorometrically using a Trilogy fluorometer fitted with the non-acidification module (CHL NA #046, Turner Designs). Subsamples for POC (1 L) and PN (2 L) were filtered onto pre-combusted GF/F filters and dried in the oven (40 °C, 48 h). Prior to analysis POC filters were decalcified with 0.1 N HCl. POC and PN filter contents were measured on a EuroVector elemental analyzer (EURO EA 3000) with analytical precision of ≤0.1% (C) and ≤0.03% (N).

Substrate collection and maintenance

Samples of the three investigated reef framework substrates; turf algae, coral rock, and the encrusting sponge *M. fistulifera*, were collected from the reef at 10 m water depth by SCUBA and transferred to the aquarium facility. Turf algae were defined as a heterogeneous assemblage of filamentous algae, crustose coralline algae (CCA), and filamentous cyanobacteria (Fricke et al. 2011). Coral rock was considered biogenic reef framework lacking coverage by a single dominant epilithic group with the carbonate structure clearly visible. In the Gulf of Aqaba this hard substrate can cover large areas of the reef and is commonly referred to as "bare rock" or "bare substrate" (eg. Shashar et al. 1994a, Bahartan et al. 2010), but is associated with endolithic algae, epilithic microbial biofilms, and sparse patches of CCA, cyanobacteria and filamentous algae (Charpy et al. 2012, Bahartan et al. 2010). *M. fistulifera* is an encrusting sponge approximately 0.2 – 0.5 cm thick, typically found encrusting coral skeletons. Turf algae and *M. fistulifera* were collected by chiseling small pieces of dead branching corals completely overgrown by either turf or *M. fistulifera* and were attached to ceramic tiles with coral glue (Reef Construct, Aqua Medic®) to minimize stress during experimental handling. Coral rock was sampled by chiseling pieces of reef framework. For each substrate, 8 replicates per season were collected with a mean height of 5-6 cm and mean surface area of 33.5 ± 18.9 cm^2. Specimens were maintained in a 1000 L flow-through tank supplied with seawater pumped directly from the reef at 10 m water depth at a rate of approximately 4000 L h^{-1}, ensuring key environmental parameters (e.g. temperature and inorganic nutrient concentrations) corresponded to seasonal *in situ* conditions. Irradiance (PAR) was adjusted to *in situ* levels at 10 m water depth using layers of black mesh. *M. fistulifera* specimens were allowed to heal and acclimate for 1 wk prior to experiments and only healthy specimens were used in incubation experiments. Turf algae and coral rock were collected 24 h before incubations were conducted.

Quantification of dinitrogen fixation

N_2 fixation rates were quantified using the acetylene (C_2H_2) reduction assay method (Capone 1993, Wilson et al. 2012). Specimens (n = 8 per substrate) were incubated in individual 1 L chambers containing 800 mL of natural seawater of which 10% was replaced with C_2H_2-saturated seawater immediately prior to the start of the incubation. Chambers were then sealed gas-tight and 10% of the 200 mL headspace was replaced with freshly generated C_2H_2 gas. Sealed chambers were stirred with magnetic stirrers (600 rpm) and incubated in the flow-through tank to ensure *in situ* temperature and irradiance throughout the 24 h incubation period. Incubations started and ended just prior to sunset (approximately 17:00) and gas samples were taken at 0, 4, 12, 16 and 24 h, except during spring when samples were taken only at 0 and 24 h. At each time interval 1 ml of gas sample was collected from the headspace of each chamber with a gastight syringe and transferred into gas-tight 2 ml glass vials fitted with butyl septa and filled with distilled water. Vials were stored frozen upside down until analysis.

Ethylene (C_2H_4) concentrations in the gas samples were measured using a reducing compound photometer (RCP) (Peak Laboratories) with a detection limit of 100 ppb. Calibration of the RCP was routinely conducted using serial dilutions of a 200 ppm (± 2 %) C_2H_4 standard in air (Restek, USA). Differences in C_2H_4 concentration between the time intervals of the incubation period were converted into C_2H_4 evolution rates according to Breitbarth et al. (2004). The C_2H_4 concentrations of the samples were corrected for the signal of unfiltered seawater controls (n = 8) and normalized to incubation time and surface area of the specimen in order to calculate C_2H_4 evolution rates (nmol C_2H_4 cm^{-2} h^{-1}). Additional controls for 0.2 µm filtered seawater (n = 6), unfiltered seawater and ceramic tile (n = 6), and unfiltered seawater with specimens but no addition of C_2H_2 (natural C_2H_4 production, n = 6), showed negligible C_2H_4 evolution. Surface areas were measured using advanced geometry (Naumann et al. 2009). To convert C_2H_4 evolution rates to N_2 fixation rates to determine the contribution of N_2 fixation to the N required for P_{gross}, a conservative theoretical ratio of 4:1 (C_2H_4:N_2) was used (Mulholland et al. 2004).

Quantification of primary productivity

Primary productivity was quantified via dissolved oxygen (O_2) fluxes. Substrates and seawater controls (n = 8 replicates each) were incubated in 1 L airtight glass chambers filled with natural seawater and sealed with a transparent glass lid. The sealed chambers were incubated under identical conditions as described above for N_2 fixation measurements. Incubations for respiration

(R) were conducted 1-2 h after sunset in complete darkness for 90 – 120 min. Incubations for net photosynthesis (P_{net}) were carried out at between 12:00 – 14:00 the following day during maximum light intensity for 60 – 90 minutes. O_2 concentrations were measured at the start and end of each incubation period using a salinity and temperature corrected O_2 optode sensor (MultiLine ® IDS 3430, WTW GmbH). Start O_2 concentrations were subtracted from end O_2 concentrations to quantify P_{net} and R. O_2 fluxes were corrected for the mean O_2 difference found in the seawater controls and normalized to incubation time and surface area of the respective specimen. Due to exceptionally low irradiances during P_{net} incubations for turf algae in spring, these values were removed from the data set. R is presented as a positive rate and gross photosynthesis (P_{gross}) rates were calculated as: $P_{gross} = P_{net} + R$.

To calculate the contribution of fixed N to the N demand for primary production, O_2 fluxes were converted into dissolved inorganic C fluxes using a photosynthetic quotient (PQ) of 1.04 and respiratory quotient (RQ) of 0.96 for turf algae and coral rock (Carpenter & Williams, 2007). Since no literature values were available for marine sponges, a PQ/RQ of 1 was used for *M. fistulifera*. It was assumed that turf algae and *M. fistulifera* assimilate biomass with C:N ratios of 13.7 ± 0.6 and 7.2 ± 0.7, respectively, based on C and N elemental analyses of macroalgae and *M. fistulifera* from the study site (Rix unpublished). Since no data were available for coral rock from the Gulf of Aqaba, C:N ratios of epi- and endolithic algae associated with coral rubble from Le Reunion and Sesoko Islands were used (9.7 ± 1.5; Casareto et al. 2008).

Statistical analysis

The influence of "season" and "substrate" on all physiological parameters was estimated using fully crossed general linear models fitted in R version 3.1.1 (R Development Core Team, 2014). A second model was run examining the effect of "season" and "substrate" and "time of day" (day or night) on N_2 fixation. Season was used as a fixed factor encompassing the combined effects of all environmental parameters. The influence of individual environmental parameters was further examined using linear regressions. To confirm the assumptions of normally distributed and homogenous residuals, qqplots and scatter plots of residuals against fitted values were visually inspected (Quinn & Keough 2002), and data were log-transformed where necessary. Model stability was checked by examining leverage and Cook's distance as well as dffits and dfbetas, and all values were deemed acceptable. Model significance was tested using likelihood ratio tests (LRT), comparing the deviances of full models with those of the null models comprising only the

intercept. The significance of individual factors was tested by removing the factor of interest and comparing the deviance to the respective full models. If factors were found to be significant, pairwise post-hoc comparisons (*t* –tests) were used to check the comparisons of interest.

Table 3.1. Environmental parameters monitored over four seasonal periods in 2013; Winter (February), Spring (April), Summer (September), Fall (November). Parameters measured include ammonia (NH_4^+), nitrate + nitrite (NOx), total dissolved inorganic nitrogen (DIN), phosphate (PO_4^{3-}), particulate organic carbon (POC), particulate nitrogen (PN), and chlorophyll a (Chl *a*). Values are presented as mean ± SD (n=4).

Parameter	Winter	Spring	Summer	Fall
Irradiance (PAR)	180 ± 43	252 ± 38	307 ± 25	171 ± 20
Temperature (°C)	22.5 ± 0.1	22.8 ± 0.3	27.5 ± 0.2	25.2 ± 0.2
NH_4^+ (μM)	0.32 ± 0.09	0.46 ± 0.11	0.14 ± 0.07	0.28 ± 0.07
NOx (μM)	0.79 ± 0.16	0.49 ± 0.19	0.09 ± 0.21	0.18 ± 0.05
DIN (μM)	1.11 ± 0.19	0.96 ± 0.08	0.23 ± 0.07	0.46 ± 0.10
PO_4^{3-} (μM)	0.11 ± 0.01	0.10 ± 0.02	0.04 ± 0.02	0.04 ± 0.02
DIN:PO_4^{3-}	10.50 ± 1.09	9.68 ± 0.43	8.10 ± 3.40	12.93 ± 2.22
POC (μM)	6.33 ± 0.70	10.25 ± 0.72	7.96 ± 1.35	8.81 ± 2.10
PN (μM)	0.85 ± 0.07	1.27 ± 0.05	0.96 ± 0.28	0.87 ± 0.37
POC:PN	7.34 ± 1.15	8.18 ± 1.29	8.34 ± 1.17	10.20 ± 1.62
Chl *a* (μg L^{-1})	0.21 ± 0.02	0.22 ± 0.04	0.10 ± 0.04	0.19 ± 0.04

Results

Environmental monitoring

All environmental parameters monitored showed marked seasonal variability over the study period, with the most pronounced differences occurring between winter and summer (Table 3.1). Irradiance was higher in spring and summer (252 and 307 μmol photons m^{-2} s^{-1}, respectively) compared to winter and fall (180 and 171 μmol m^{-2} s^{-1}, respectively). Temperature ranged from 22.4 to 28.0 °C over the year, remaining low throughout winter and spring then reaching a maximum in summer before decreasing again in fall (Table 3.1). Concentrations of NH_4^+, NO_x and PO_4^3

exhibited a negative correlation with temperature (lm: all $p < 0.001$), with concentrations more than twice as high in winter and spring compared to summer and fall, reflecting the deep winter mixing and summer stratification of the water column. The ratio of dissolved inorganic nitrogen to phosphate (DIN:PO_4^3) ranged from 3.4 to 15.6 over the year but was consistently lower than the Redfield ratio (16:1), indicating N limitation in the water column. This was further suggested by POC:PN ratios that always exceeded the Redfield ratio (106:16). Chl *a* concentrations decreased by half in summer compared to all other seasons, while concentrations of POC and PN were highest in spring during the seasonal plankton bloom (Table 3.1).

Dinitrogen fixation

N_2 fixation activity varied significantly by substrate, season, and an interaction between the two factors (Fig. 3.1, Supplementary Table 3.S1). On annual average, N_2 fixation was significantly higher in turf algae (4.4 ± 3.9 nmol C_2H_4 cm^{-2} h^{-1}) and coral rock (3.5 ± 2.8 nmol C_2H_4 cm^{-2} h^{-1}) compared to *M. fistulifera* (0.2 ± 0.2 nmol C_2H_4 cm^{-2} h^{-1}) (post hoc paired *t*-test: both $p<0.001$). N_2 fixation for turf algae and coral rock was similar on annual average but significantly higher for turf algae in winter and summer, although in summer this was entirely due to higher nighttime N_2 fixation in turf algae (post hoc paired *t*-test: all $p<0.001$). N_2 fixation rates for all substrates were significantly higher in summer compared to all other seasons (post hoc paired *t*-test: all $p<0.001$) (Fig. 3.1). Coral rock and *M. fistulifera* also displayed significantly lower N_2 fixation activity in winter compared to other seasons (post hoc paired *t*-test: all $p<0.05$). There were no significant differences in N_2 fixation between spring and fall for any substrate (Fig. 3.1). Across all substrates, N_2 fixation was one order of magnitude higher in the summer compared to the lowest rates in winter. Irradiance and temperature had a positive effect on N_2 fixation, while inorganic nutrient concentrations had a negative effect (Table 3.2). Irradiance explained more variation in N_2 fixation in turf algae and *M. fistulifera* than in coral rock, and for all substrates DIN concentrations explained more variation in N_2 fixation than PO_4^{3-} concentrations (Table 3.2).

N_2 fixation activity also varied significantly by time of day (Fig. 3.2, Supplementary Table 3.S2). *M. fistulifera* exhibited significantly higher N_2 fixation during the day compared to the night in all three seasons examined (i.e. winter, summer, and fall) (post hoc paired *t*-test: all $p < 0.01$). N_2 fixation in turf algae showed a seasonally variable response to time of day, with significantly higher daytime N_2 fixation in winter but significantly higher nighttime N_2 fixation in summer (Fig. 3.2). This was the only instance of significantly higher N_2 fixation at night but it was also the highest N_2

fixation rate measured over all substrates and seasons, with a rate of 17.8 ± 5.5 nmol C_2H_4 cm^{-2} h^{-1}. Coral rock displayed no significant differences in N_2 fixation between day and night (Fig. 3.2).

Table 3.2. Linear regression analysis of key environmental parameters and N_2 fixation rates in the three substrates. Data presented as R-squared values at significance levels of <0.05*, <0.01**, <0.001***. Italicized values indicate a significant positive linear relationship and bold values indicating a significant negative linear relationship. Abbreviations: P_{gross} = gross photosynthesis, DIN = dissolved inorganic nitrogen, PO_4^{3-} = phosphate.

		Irradiance	**Temperature**	**DIN**	**PO_4^{3-}**
N_2 fixation	Turf algae	*0.542****	*0.696****	**0.586*****	**0.399*****
	Coral rock	*0.415****	*0.458****	**0.511*****	**0.396*****
	Mycale fistulifera	*0.503****	*0.714****	**0.696*****	**0.584*****
P_{gross}	Turf algae	*0.505****	*0.424****	**0.305****	**0.163 ***
	Coral rock	0.028 NS	0.122 NS	0.096 NS	0.088 NS
	Mycale fistulifera	*0.403****	*0.212***	**0.244****	**0.162***

Table 3.3. Metabolic parameters measured in the three substrates over four seasonal periods. Rates are presented as nmol O_2 cm^{-2} h^{-1} (mean ± SD, n=8). Abbreviations: P_{gross} = gross photosynthesis, R = respiration, P_{net} = net photosynthesis.

Substrate	**Season**	**P_{gross}**	**R**	**P_{net}**	**P_{gross}:R**
Turf algae	Winter	528 ± 85	95 ± 12	433 ± 77	5.6 ± 0.7
	Spring	NA	78 ± 19	NA	NA
	Summer	894 ± 162	119 ± 27	77 ± 155	7.8 ± 2.1
	Fall	509 ± 84	95 ± 19	415 ± 68	5.4 ± 0.6
	Mean	**620 ± 195**	**97 ± 24**	**524 ± 179**	**6.5 ± 1.6**
Coral rock	Winter	472 ± 237	96 ± 55	379 ± 186	4.7 ± 0.4
	Spring	354 ± 165	69 ± 378	271 ± 136	3.4 ± 1.9
	Summer	553 ± 152	179 ± 95	374 ± 124	3.5 ± 1.2
	Fall	446 ± 134	144 ±35	302 ± 105	3.1 ± 0.5
	Mean	**438 ± 189**	**122 ± 72**	**316 ± 158**	**3.7 ± 1.9**
Mycale fistulifera	Winter	64 ± 49	238 ± 38	-174 ± 27	0.3 ± 0.1
	Spring	220 ± 42	348 ± 103	-139 ± 109	0.7 ± 0.2
	Summer	307 ± 108	563 ± 106	-256 ± 80	0.5 ± 0.2
	Fall	139 ± 68	564 ± 106	-393 ± 190	0.3 ± 0.2
	Mean	**190 ± 121**	**431 ± 164**	**-240 ± 149**	**0.5 ± 0.3**

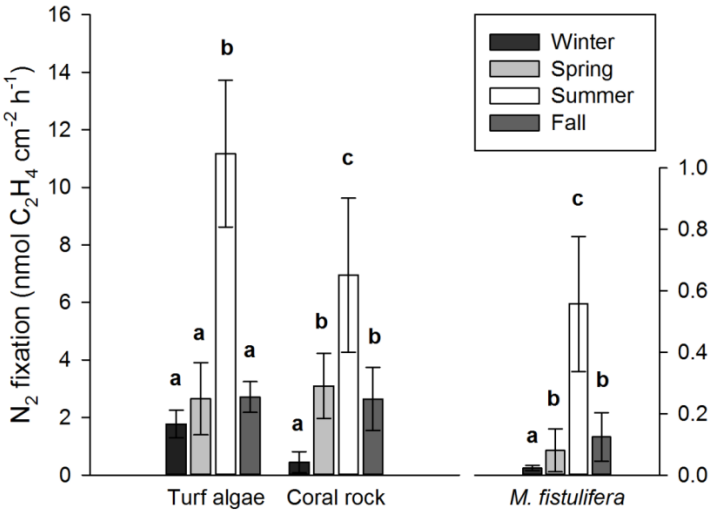

Figure 3.1. Mean N_2 fixation rates of the three investigated benthic substrates over the four seasonal periods in 2013; winter (February), spring (April), summer (September), fall (November). Values (acetylene reduction nmol C_2H_4 cm^{-2} h^{-1}) are presented as mean (n = 8) ± SD. Letters indicate statistical differences within each substrate. Note the different y-axis scale for *Mycale fistulifera*.

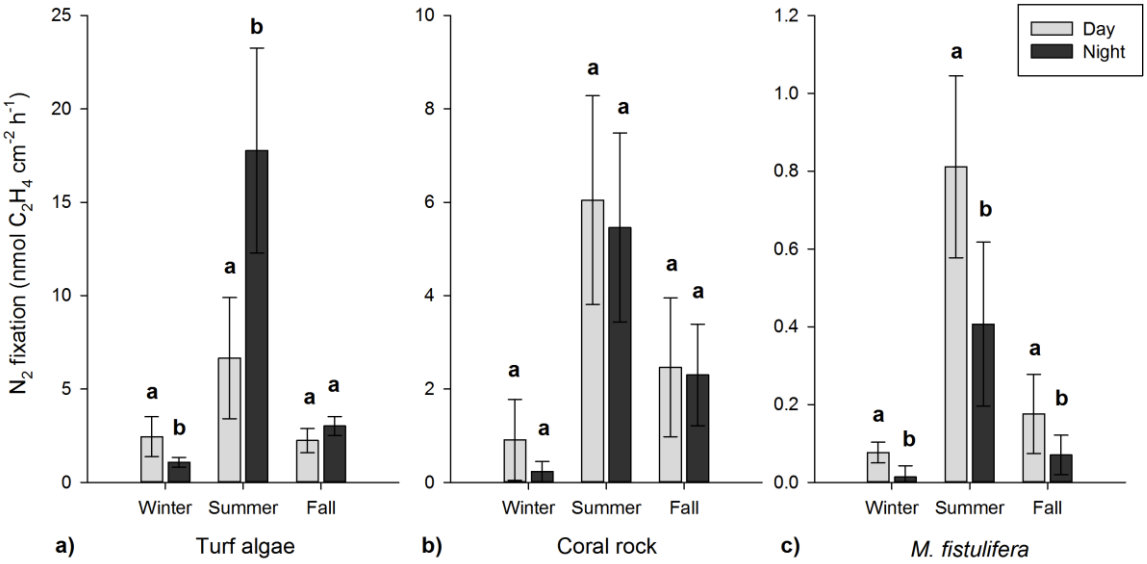

Figure 3.2. Mean day (light bars) and night (dark bars) N_2 fixation rates of a) turf algae, b) coral rock, and c) *Mycale fistulifera* measured over three seasons in 2013 (winter, summer, and fall). Values (acetylene reduction nmol C_2H_4 cm^{-2} h^{-1}) are presented as mean (n = 8) ± SD. Letters indicate statistical differences within each substrate. Note the changes in scale of the y-axis.

Primary productivity

There were significant effects of substrate and season as well as significant interactions between the two factors for all physiological parameters measured (Supplementary Table 3.S1). Over all seasons, rates of P_{gross} were significantly higher in turf algae compared to coral rock (post hoc paired *t*-test: $p < 0.001$) and significantly lower in *M. fistulifera* compared to both other substrates (post hoc paired *t*-test: both $p < 0.001$). Despite calculated positive P_{gross} rates, *M. fistulifera* exhibited no P_{net} (Table 3.3). This was due to high R rates, which were significantly higher than for turf algae and coral rock (post hoc paired *t*-test: both $p < 0.001$) and resulted in low P_{gross}:R ratios (< 1). Coral rock exhibited significantly higher R and lower P_{gross}:R ratios than turf algae (post hoc paired *t*-test: both $p < 0.001$) (Table 3.3).

Seasonal variations in P_{gross} were less pronounced than the seasonal differences in N_2 fixation (Table 3.3). P_{gross} was significantly higher in turf algae in summer (post hoc paired *t*-test: all $p < 0.001$) and significantly lower in coral rock in spring (post hoc paired *t*-test: $p < 0.01$) compared to other seasons (Table 3.3). P_{gross} in *M. fistulifera* was significantly higher in spring compared to winter and in summer compared at all other seasons (post hoc paired *t*-test: all $p < 0.001$). Irradiance explained the most variation in P_{gross} in turf algae and *M. fistulifera* (Table 3.2). There was little seasonal variation in P_{gross} of coral rock with no significant effect of any of the monitored environmental parameters (Table 3.2).

Contribution of dinitrogen fixation to primary productivity

There was a significant positive linear relationship between P_{gross} and N_2 fixation for turf algae and *M. fistulifera*, with N_2 fixation rates explaining 81% of the variation in P_{gross} in turf algae and 37% in *M. fistulifera* (Fig. 3.3). In contrast there was no significant relationship between N_2 fixation and P_{gross} in coral rock. The annual average contributions of N_2 fixation to the N demand for P_{gross} in turf algae, coral rock, and *M. fistulifera* were calculated to be 8.9, 7.1, and 0.6%, respectively (Table 3.4). This contribution was seasonally variable for all substrates with N_2 fixation supplying the highest amounts of N in summer (up to 12.8 and 17.2% for coral rock and turf algae, respectively), while the contributions in winter were less than 5% for all substrates (Table.3. 4).

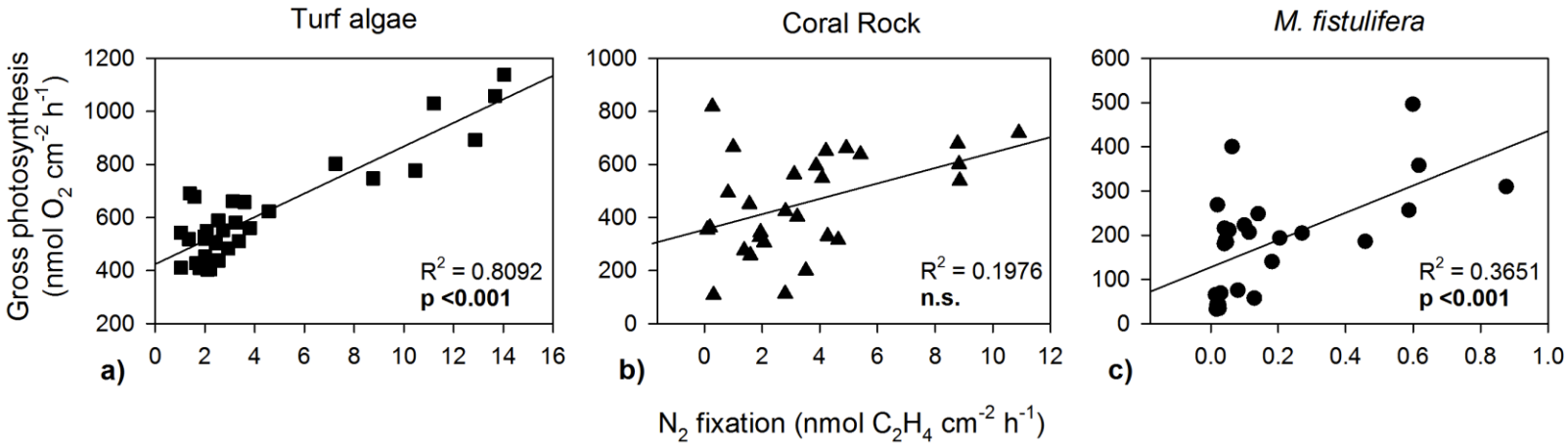

Figure 3.3. Linear regression analysis of N_2 fixation and gross photosynthesis (P_{gross}) rates over all seasons in a) turf algae, b) coral rock, and c) *Mycale fistulifera*. N_2 fixation is presented as acetylene reduction (nmol C_2H_4 cm^{-2} h^{-1}) and P_{gross} is presented as O_2 fluxes (nmol O_2 cm^{-2} h^{-1}).

Table 3.4. Nitrogen (N) demand of gross primary productivity (P_{gross}) in the three investigated substrates and the percentage contributions of N fixed by N_2 fixation. Values are reported as mean ± SD (n = 8).

Substrate	Season	N demand for P_{gross} (µmol N cm^{-2} d^{-1})	Contribution of fixed N to P_{gross} (%)
Turf algae	Winter	0.44 ± 0.07	4.8 ± 1.1
	Spring	NA	NA
	Summer	0.75 ± 0.13	17.2 ± 2.5
	Fall	0.43 ± 0.07	7.6 ± 0.8
	Mean	**0.52 ± 0.17**	**8.9 ± 5.1**
Coral rock	Winter	0.56 ± 0.30	1.2 ± 1.0
	Spring	0.42 ± 0.20	6.9 ± 1.0
	Summer	0.66 ± 0.18	12.8 ± 3.5
	Fall	0.53 ± 0.16	5.9 ± 1.3
	Mean	**0.55 ± 0.22**	**7.1 ± 4.9**
Mycale fistulifera	Winter	0.13 ± 0.10	0.3 ± 0.1
	Spring	0.54 ± 0.23	0.2 ± 0.1
	Summer	0.61 ± 0.22	1.4 ± 0.7
	Fall	0.34 ± 0.22	0.7 ± 0.6
	Mean	**0.40 ± 0.27**	**0.6 ± 0.6**

Discussion

Dinitrogen fixation in coral reef framework substrates

The N_2 fixation rates presented here for the three investigated reef framework substrates are comparable to those reported for turf algae, coral rock, and sponges on coral reefs worldwide (Table 3.5). The relatively high variability in N_2 fixation rates reported for turf algae likely results from regional differences in diazotroph community composition (Bauer et al. 2008) and responses to local environmental conditions (Carpenter et al. 1991, Williams & Carpenter 1997). While N_2 fixation rates reported for turf algae by previous studies are typically higher than those reported for coral rock (Table 3.5), our annual average rates for both substrates were similar, with significantly higher N_2 fixation in turf algae only during winter and summer at night. However, studies reporting higher rates of N_2 fixation in "bare" coral rock compared to rock with epilithic algal, suggest endolithic N_2 fixation can exceed that of some epilithic communities (Wilkinson et al. 1984, Casareto et al. 2008). This highlights the role of apparently "bare" substrate in generating new N on coral reefs.

N_2 fixation rates in *M. fistulifera* were an order of magnitude lower than for turf algae and coral rock, but are in the range of other animal-microbe symbioses such as scleractinian corals (Shashar et al. 1994b, Davey et al. 2008) and other cnidarians (Shashar et al. 1994a,). Further, the N_2 fixation rates for *M. fistulifera* are consistent with those reported for other Red Sea sponges in the only other study presenting sponge N_2 fixation rates normalized to organism surface area (Shashar et al 1994a, Table 3.5). The lower N_2 fixation activity in *M. fistulifera* compared to turf algae and coral rock is likely because, as efficient filter feeders, sponges are less dependent on N_2 fixation as a source of N (Pile et al. 2003). This is supported by the low P_{gross}:R ratios in *M. fistulifera* (< 1), which indicate heterotrophic metabolism (Wilkinson 1987).

Table 3.5. Comparison of known N_2 fixation rates of turf algae, coral rock, and sponges reported from coral reefs worldwide. Values are presented as nmol N cm^{-2} h^{-1}. Original C_2H_4:N_2 conversion rates were used to calculate the N_2 fixation rates from acetylene reduction rates if reported in the original study. If no conversion rate was available the conservative ratio of 4:1 was used.

Substrate	N fixation	Region	Reference
Turf algae	0.9 – 5.6	Red Sea	Present study
Turf algae	4.6 ± 0.3	Red Sea	Shashar et al. 1994a
Turf algae	0.3 – 29.7[a]	Great Barrier Reef	Larkum et al. 1988
Turf algae	8.3 – 36.7	Great Barrier Reef	Wilkinson & Sammarco 1983
Turf algae	13.5 ± 5.5	Hawaiian Islands	Williams & Carpenter 1998
Turf algae	3.7 ± 5.4	Caribbean	Williams & Carpenter 1997
Turf algae	6.0 ± 0.9	Caribbean	Den Haan et al. 2014
Coral rock	0.2 – 3.5	Red Sea	Present study
Coral rock	0.3 ± 0.2	Red Sea	Shashar et al. 1994a
Coral rock	0.2 – 1.9	Great Barrier Reef	Wilkinson et al. 1984
Coral rock	0.1 – 6.4	Great Barrier Reef	Davey et al. 2008
Coral rock	6.4 ± 1.8[a]	Great Barrier Reef	Larkum et al. 1988
Coral rock	0.6[b]	French Polynesia	Charpy-Roubaud et al. 2001
Sponge (*M. fistulifera*)	0.01 – 0.3	Red Sea	Present study
Sponges	0.1 ± 0.2	Red Sea	Shashar et al. 1994a

[a] Conversion factor 3.45
[b] Conversion factor 3.3

Diel dinitrogen fixation pattern

While many studies have found substantially higher daytime N_2 fixation activity in coral rock (Wilkinson et al. 1984, Charpy-Roubaud et al. 2001, Holmes & Johnstone 2010) and turf algae (Williams & Carpenter 1997, Den Haan et al. 2014), our results show consistent and substantial nighttime N_2 fixation by both substrates. Turf algae exhibited significantly higher daytime N_2 fixation in winter but significantly (3-times) higher nighttime N_2 fixation in summer, which may indicate a shift in the turf community towards more non-heterocystous cyanobacteria in summer, as few non-heterocystous cyanobacteria can fix N_2 in the presence of O_2 generated by photosynthesis (Bergman et al. 1997). Similar day and night N_2 fixation rates in coral rock suggest a balanced diazotroph community composed of heterocystous and non-heterocystous cyanobacteria or heterotrophic bacteria (Diez et al. 2007). Cyanobacteria have long been considered the primary diazotrophs responsible for benthic marine N_2 fixation, but the role of heterotrophic bacteria is increasingly being recognized (Zehr et al. 1995, Bauer et al. 2008). Only *M. fistulifera* consistently exhibited significantly higher N_2 fixation activity in the day compared to night (2 times higher), suggesting the role of phototrophic symbionts. Indeed, symbiotic cyanobacteria are widespread in coral reef sponges (Wilkinson & Fay 1979, Erwin & Thacker 2007), and the expression of cyanobacterial *nifH* genes has been detected in the congeneric *Mycale laxissima* from Florida (USA) (Mohammed et al. 2008). Likely, photosynthetic activity of cyanobacterial symbionts is also responsible for our findings of P_{gross} in *M. fistulifera*. Despite exhibiting higher daytime N_2 fixation, *M. fistulifera* also exhibited nighttime N_2 fixation activity suggesting a continued supply of fixed N throughout the day and night in all three substrates.

Seasonality in dinitrogen fixation and primary productivity

This is the first study examining seasonal N_2 fixation in a diverse group of reef framework substrates, and our findings highlight the importance of key environmental parameters in regulating benthic N_2 fixation activity. The pronounced seasonal variation in environmental parameters is reflected by the N_2 fixation activity in the three substrates, with highest rates in summer when irradiance and temperature are highest but inorganic nutrients lowest. This seasonal pattern shows remarkable consistency across the three groups despite their differing trophic strategies. Primary productivity in coral rock shows less pronounced seasonal variability, but turf algae and *M. fistulifera* both exhibit significantly higher P_{gross} in summer, driven primarily by higher irradiance. Irradiance may likewise stimulate the energetically costly process of N_2 fixation through the provision of larger

quantities of energy-rich photosynthates (Bebout et al. 1993), which likely explains the positive effect of irradiance on N_2 fixation in the three substrates. Temperature also had a positive effect on N_2 fixation, which is consistent with measurements of reduced N_2 fixation rates in the GBR during lower winter temperatures (Larkum et al. 1988). Higher temperatures are associated with increased growth and N_2 fixation in some free-living cyanobacteria (Breitbarth et al 2007), while lower temperatures can increase respiratory costs associated with N_2 fixation in unicellular cyanobacteria (Brauer et al. 2013), which may explain this effect. The negative effect of increased DIN concentrations on N_2 fixation may be explained by the higher energetic costs of N_2 fixation compared to DIN assimilation (Gallon 2001), making it a seasonal strategy when external nutrients are scarce. This is supported by observations of increased nitrogenase activity in N-starved cultured filamentous cyanobacteria (Ramos et al. 1985) and inhibition of N_2 fixation in coral skeletons and reef sediments under elevated NH_4^+ concentrations (Koop et al. 2001, Holmes & Johnstone 2010), which suggest diazotrophs are capable of altering their N_2 fixation activity to adjust to the availability of external N sources. Alternatively, seasonal variability in N_2 fixation activity may reflect microbial community changes as diazotrophs may be more competitive than non-fixing microorganisms under low nutrient conditions. Such competitive interactions have been observed for plankton communities (Agawin et al. 2007) and microbial mats (Yannarell et al. 2006). Overall the combination of key environmental parameters in summer (i.e. high irradiance, high temperature, and low inorganic nutrients) appear to interact to cause the substantially higher N_2 fixation rates found for all three substrates.

Contribution of dinitrogen fixation to primary productivity

Interestingly, higher P_{gross} rates were sustained in summer despite lower inorganic nutrient availability. This may be explained by the highly significant linear relationship between N_2 fixation and P_{gross} in turf algae, which suggests tight coupling between the two processes. We estimated fixed N could supply 4.7% of the N demand for P_{gross} in winter and 17.1% in summer, suggesting N_2 fixation is an important N supply for photosynthesis. By contrast, Williams & Carpenter (1997) found N_2 fixation contributed less than 2% to the N demand for P_{net} in turf algae in the Caribbean, with the estimated contribution by NH_4^+ assimilation an order of magnitude higher. While DIN assimilation likely represents an important process at our study site, increased summer N_2 fixation may compensate for decreased DIN concentrations allowing the turf community to achieve 1.7 times higher P_{gross} rates. While N_2 fixation and P_{gross} are also correlated in *M. fistulifera*, N_2 fixation explains much less variation in P_{gross}, suggesting a weak coupling between the two processes within

the sponge. Further, fixed N supplies less than 1.5% of the N required to support P_{gross} in *M. fistulifera*. Given that *M. fistulifera* exhibited no P_{net} and lower N_2 fixation rates, these processes are likely less important to the overall nutrition of *M. fistulifera*. There was no significant relationship between P_{gross} and N_2 fixation in coral rock. Lower P_{gross}:R ratios in coral rock compared to turf algae suggest increased heterotrophy, and increased heterotrophic N_2 fixation could explain the lack of correlation between the two processes. However, coral rock was overall net photosynthetic and we estimate that N_2 fixation could supply 1.2 % of the N demand for P_{gross} in winter and 12.8 % in summer. These estimates are consistent with those from Sesoko Island (Pacific Ocean) and Le Reunion (Indian Ocean) where N_2 fixation in coral rock contributed 4 – 10% and 2 – 17%, respectively, to primary production (Casareto et al. 2008). However, our estimates likely underestimate the importance of fixed N, as recycling of N within the substrates may dramatically reduce the actual demand of P_{gross} for new N (Suzuki et al. 1995, Charpy-Roubaud et al. 2001) and consequently increase the relative contribution by N_2 fixation. Overall, these findings suggest N_2 fixation strongly contributes to sustaining high rates of P_{gross} in coral rock and turf algae during the nutrient depleted summer season in the Gulf of Aqaba.

Ecological implications

In addition to directly supporting their own growth, the high rates of N_2 fixation in turf algae and coral rock may substantially contribute to new N on coral reefs via the release of organic and inorganic dissolved N from cyanobacterial cells (Mulholland et al. 2006) or by mechanical disturbances such as grazing (Williams & Carpenter 1997). Given the low abundance of sponges on the studied reef and the comparatively lower rates of N_2 fixation of *M. fistulifera*, the sponge community likely does not contribute substantially to new N on the reef, at least via N_2 fixation. Sponges can produce large quantities of inorganic nutrients through remineralization of particulate organic matter and nitrification (Southwell et al. 2008). While turf algae are the dominant component on many coral reefs (e.g. Bahartan et al. 2010), their low and seasonally variable abundance in the present study site suggests only a minor contribution to reef-wide primary productivity and N generation, despite high P_{gross} and N_2 fixation rates. High N_2 fixation and high benthic coverage (11 - 20%) suggest coral rock is the largest contributor of newly fixed N among the three investigated substrates. This fixed N appears to be of particular ecological importance during the low nutrient summer season in the Gulf of Aqaba, suggesting the potential importance of N_2 fixation in coral rock and turf algae, particularly in supporting primary production, in coral reef ecosystems with more constant oligotrophic conditions.

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Chapter IV

Microbial dinitrogen fixation sustains high coral productivity in oligotrophic reef ecosystems*

Abstract

In nutrient-depleted waters, hard corals act as primary ecosystem engineers of tropical reefs by providing the structural complexity, biomass, and organic matter on which the entire reef ecosystem is sustained. The exceptionally high gross primary production of corals is achieved by efficient recycling of nutrients through coordinated metabolic exchanges with their endosymbiotic dinoflagellate algae (zooxanthellae). However, additional inputs of nitrogen are required for net production and growth. Here we show that photoautotrophic inorganic carbon (CO_2) fixation by zooxanthellae is sustained by dinitrogen (N_2) fixation performed by coral-associated N_2-fixing prokaryotes if other external sources of inorganic nutrients are scarce. The functioning of this host-microbe ecosystem enables the nutritional success of hard corals and explains their high gross productivity and organic matter release, which in turn set the basis for the functioning of reef ecosystems and the services that they provide.

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Introduction

Scleractinian hard corals fulfill important functions as key ecosystem engineers of tropical coral reefs, providing the habitat for some of the most productive, diverse and economically important biological systems on Earth[1]. They do so by controlling a wide range of biogeochemical processes important to coral reef functioning and by intensively generating and transforming inorganic and organic materials[2]. These sessile cnidarians owe their evolutionary success to an endosymbiosis with photosynthetic dinoflagellate microalgae of the genus *Symbiodinium* (commonly referred to as zooxanthellae)[3, 4] that contribute a substantial fraction of the total gross primary productivity (i.e., the amount of inorganic carbon (CO_2) photosynthetically fixed per unit of time) in coral reef ecosystems. Up to 50% of the net fixed CO_2 in the coral is subsequently released as organic carbon (C) in the form of mucus, and provides a nutritious food source to other reef organisms via the microbial and sponge loops[5, 6].

Hard corals show a remarkable degree of nutritional plasticity, being able to fix CO_2 at high rates, but also adapt and rapidly assimilate organic C when zooxanthellae are lost (e.g., during coral bleaching)[7]. Additionally, corals have evolved strategies to exploit any source of inorganic and organic nitrogen (N)[8], enabling them to survive in oligotrophic tropical waters where N is the most limiting nutrient to primary productivity[9, 10]. However, the abundance of symbiotic zooxanthellae within the coral host is limited by N concentrations[4, 11]. N deficiency and starvation of zooxanthellae cause severe depletion of the main N-containing photopigment[12, 13], chlorophyll *a* (chl *a*), and result in a decline in productivity. Although very efficient internal recycling of N prevents disintegration of the symbiosis and loss of zooxanthellae, recycled N cannot account for new biomass production and growth if external sources are in short supply[14].

Besides endosymbiotic eukaryotic algae, hard corals are also associated with a diverse array of prokaryotes[15]. The prokaryote-dinoflagellate-coral assemblage is often referred to as the coral holobiont[15]. In the resulting host-microbe ecosystem, the interactions among the partners determine the functioning and ecological success of the whole group of organisms[16]. Evidence is accumulating that among these prokaryotic communities dinitrogen (N_2) fixing microorganisms (termed diazotrophs) form highly specific associations with their cnidarian hosts[17, 18, 19, 20, 21, 22]. However, the ecological significance of N_2 fixation in sustaining the high gross primary productivity of hard corals has not yet been evaluated.

Here, we present results of a year-long study examining the relationship between N_2 fixation and CO_2 fixation in hard coral holobionts at a fringing coral reef in the northern Red Sea

(Gulf of Aqaba). We made use of the characteristic seasonality of the sampling location, which harbors some of the northernmost warm water coral reefs on Earth. At our study location, water column mixing takes place during spring and strong thermal stratification occurs in summer (Fig. 4.1, Supplementary Fig. 4.S1). This leads to inorganic N concentrations which are one order of magnitude lower in summer compared to spring (Supplementary Table 4.S1). We thus repeated our measurements of the four locally dominant hard coral genera during all seasons. This strategy allowed us to assess the contribution of diazotrophs to changing N requirements of the coral holobiont in response to pronounced seasonal environmental variations.

Methods

Collection and maintenance of corals

All research described was conducted at the northern Red Sea (Gulf of Aqaba) during 2013. Sample collection was carried out along the slope of the fringing reef in front of the Marine Science Station (MSS) Aqaba, Jordan (location: 29° 27' N, 34° 58' E) using SCUBA. In February (winter), April (spring), September (summer) and November (autumn), 8 coral fragments (5-6 cm in height) of each of the hard coral genera *Acropora*, *Pocillopora*, *Stylophora* and *Goniastrea*, identified morphologically, were sampled haphazardly at 10 m water depth from individual colonies that were at least 5 m apart to avoid sampling clones. Fragments were cut underwater with bone cutters and placed in plastic bags, taking care not to cause any abrasion of the tissues. In the laboratory, fragments were glued on ceramic tiles and maintained in a 1000 L flow-through aquarium directly supplied with untreated reef water (from 10 m water depth) at a rate of approximately 4000 L h^{-1}. Natural levels of light intensity inside the maintenance aquarium were adjusted to seasonal *in situ* conditions at 10 m water depth using layers of black mesh. Monthly measurements of light intensity and inorganic nutrient concentrations in the flow-through aquarium were performed and differences from *in situ* conditions were undetectable.

Environmental monitoring

Temperature and light intensity were continuously monitored at the coral sampling location on the reef (10 m depth) during all seasons by means of data loggers (Onset HOBO Pendant UA-002-64; Bourne, MA, USA). Monthly measurements of light intensity were also performed using a

quantum sensor (Model LI-192SA; Li-Cor, Lincoln, Nebraska, USA) parallel to data loggers to allow conversion from light intensity (LUX) to photosynthetically active radiation (PAR, μmol photons m^{-2} s^{-1}, wavelength 400 to 700 nm)[31]. Seawater inorganic nutrients, chlorophyll *a* (chl *a*), particulate organic C (POC), particulate N (PN) and dissolved organic C (DOC) concentrations were monitored weekly *in situ* at the coral sampling location. Seawater samples (n=4) were collected by SCUBA approximately 1 m above the substrate, using high-density polyethylene canisters (6 L). In the laboratory, subsamples were drawn from each canister for analyses of inorganic nutrients, chl *a*, POC, PN and DOC as detailed in the following. For each inorganic nutrient analysis, one subsample of 50 mL was collected from each canister (n=4) and gently filtered through cellulose acetate membrane filters (nominal pore size: 0.45 μm). Immediately after filtration, inorganic nutrient concentrations (ammonium - NH_4^+, phosphate - PO_4^{3-}, nitrate - NO_3^-, nitrite - NO_2^-) were measured using established methods[32-34]. NH_4^+ was determined fluorometrically using a Trilogy fluorometer (Turner Designs), while all other inorganic nutrients were measured spectrophotometrically with a V-630 UV-Vis Spectrophotometer (Jasco Analytical Instruments). Detection limits for NH_4^+, PO_4^{3-}, NO_x and NO_2^- were 0.09, 0.01, 0.02 and 0.005 μM, respectively. For chl *a*, one subsample of 1 L was collected from each canister (n=4), filtered onto a pre-combusted (450°C, 4h) GF/F filter (VWR, diameter: 25 mm, nominal pore size 0.7 μm), and frozen at -80°C in the dark until analysis. Prior to analysis, chl *a* was extracted with 90% acetone for 12 h in the dark at 4°C, and analysed on the Trilogy fluorometer fitted with the non-acidification chl module (CHL NA #046). For POC and PN analyses, subsamples of 1 and 2 L, respectively, were collected from each canister (n=4), filtered onto pre-combusted GF/F filters, dried for 48 h at 40°C, and stored dry until analysis. POC filters were acidified (0.1N HCl) prior to analysis, and POC and PN content of dried filters were measured using a EuroVector elemental analyser (EURO EA 3000). C and N content were derived from calculations using elemental standards (OAS 187560; analytical precision ≤0.1% (C) and ≤0.03% (N) of the standard value). For DOC analyses, one 50 mL subsample was collected from each canister (n=4), gently vacuum filtered (max. suction pressure 20 kPa) through a pre-combusted GF/F filter directly into acid-washed 30 mL HDPE sample bottles using a custom set-up with an in-line polycarbonate filter holder. Prior to filtration, the syringes, the HDPE sample bottles and the filtration set-up were soaked in 0.4M HCl for 24 h and then washed with MQ water. Upon collection, the first 20 mL of sample was used to wash the filtration set-up and the sample bottle with 2 x 10 mL sample water and discarded before collecting the remaining 30 mL. DOC samples were immediately acidified with 80 μl of 18.5% HCl and stored in the dark at 4°C until analysis. Samples were analysed by the high-temperature catalytic oxidation (HTCO) method on a Shimadzu TOC-V_{CPH} total organic carbon analyser. The

instrument was calibrated with a 10-point calibration curve of serial dilutions from a potassium hydrogen phthalate certified stock solution (1000 ppm Standard Fluka 76067-500ML-F). Consensus reference material provided by DA Hansell and W Chan of the University of Miami (Batch 13, Lot #08-13, 41-45 μmol C L^{-1}) was used a positive control between every 10 samples. Each sample was measured with 5 replicate injections, and analytical precision was <3% of the certified value.

Physiological measurements

Coral fragments (n=8 for each coral genus during each season) were maintained in the flow-through aquarium described above during measurements of net photosynthesis (Pn), dark respiration (R), N_2 fixation and POC and DOC release to ensure *in situ* temperature and light intensity in the chambers. Fragments were allowed to recover from fragmentation for at least one week prior to the measurements. Ceramic tiles were cleaned of sediment and epibionts with a fine brush immediately prior to the start of the incubations. Only visually healthy and entirely healed fragments were used for incubation experiments. Incubation chambers were filled with the seawater from the aquarium and corals were transferred into the chambers, taking particular care to prevent any air exposure. Before closing the chamber for incubation, each fragment was allowed to adjust to chamber conditions for at least 15 min. Each chamber contained a stir bar powered by a submersible magnetic stirrer (600 rpm, Cimarec i Telesystem Multipoint Stirrers, Thermo Scientific) to ensure water mixing (and gas equilibration with the headspace, if applicable). Temperature and light intensity were monitored during all measurements by placing a data logger in one additional chamber. Following each incubation, the dissolved oxygen (O_2) concentration in the incubation water was measured to ensure that oxic conditions in the chambers were maintained. Rates of Pn, R, N_2 fixation, organic matter (OM) release as well as zooxanthellae density and chlorophyll *a* (chl *a*) content were normalized to skeletal surface area of the coral fragments, measured using the Advanced Geometry protocol[35].

Primary productivity

Pn and R of the coral fragments were assessed by their O_2 fluxes in closed-cell respirometric glass chambers (1 L). Gross photosynthesis (Pg) was calculated (Pg=Pn+R) for each specimen. R was measured in the dark, one hour after sunset, to allow for specimens to reach a stable dark respiration rate. The following day, Pn was measured between 12:00 and 14:00, the most

stable and maximum light conditions of the day as confirmed by light intensity monitoring. The Pg rates presented here should therefore be considered a conservative estimate. Coral O_2 fluxes were determined as differences between salinity corrected O_2 start and end concentrations (incubation time ≈ 90 min) measured with an O2 optode sensor (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). Two different sets of controls were incubated in parallel to determine O_2 fluxes in the same unfiltered seawater and unfiltered seawater plus the mounting tile. Differences in O_2 concentration were corrected for control signals (absolute blank/biological ratios < 20%), normalized to incubation period and fragment surface area and reported as μg O_2 cm^{-2} h^{-1}. All coral fragments were returned to the maintenance aquarium for approximately 4 h before starting subsequent incubation measurements for N_2 fixation rates.

N_2 fixation

N_2 fixation was quantified by using an adapted acetylene (C_2H_2) reduction technique[36,37]. For this purpose, 1 L glass chambers were filled with 800 mL of natural seawater, whereof 10% was replaced with C_2H_2-saturated seawater. The chamber was immediately sealed gas-tight and 10% of the 200 mL headspace was replaced with C_2H_2 gas through a gas-tight rubber stopper equipped with a push-button syringe valve with Luer lock (SGE Analytical Science). To generate the C_2H_2 gas and the C_2H_2-saturated seawater, calcium carbide (CaC_2; Sigma) was used following a modified procedure described in Wilson, *et al.*[36]. Briefly, C_2H_2 was bubbled in an additional flask with 300 mL of deionized water to reduce background ethylene (C_2H_4) contamination, before being bubbled in a flask filled with 800 mL of natural seawater to be enriched. From there, the C_2H_2 was finally collected in a Tedlar® gas sampling bag. C_2H_2 and the C_2H_2-saturated seawater were freshly prepared ≈10 min prior to use. N_2 fixation incubations lasted for 24 h, starting and ending before sunset (approximately at 17:00). The incubation time was kept shorter than in previous studies on corals[22], in order to minimize stress for the organisms. Gas samples (1 mL) were withdrawn from the headspace after time intervals of 0, 4, 12, 16 and 24 h and collected in glass vials with grey butyl stoppers (CS-Chromatographie Service GmbH) previously filled with deionized water. Vials were stored frozen upside down until analysis to prevent any leakage via the septa. Prior to analysis, vials were thawed and water and gas phases in the vials were equilibrated at room temperature by vigorous shaking. C_2H_4 concentration in the vials' headspace was measured using a reducing compound photometer (RCP; Peak Laboratories)[36]. The instrument was customized with a sample loop of 10 μL which resulted in an optimal compromise between high sensitivity (± 100 ppb) and wide linear range. Calibration of the RCP was routinely conducted using serial dilutions of a 200

ppm (± 2 %) C_2H_4 standard in air (Restek, Bellefonte, PA, USA). Here, differences in C_2H_4 concentration between the time intervals of the incubation period were converted into daily C_2H_4 evolution rates according to Breitbarth, *et al.*[38]. Four sets of controls were also tested for C_2H_4 production: 1) 0.2 μm-filtered seawater, 2) unfiltered seawater, 3) unfiltered seawater plus the tile and 4) unfiltered seawater with coral fragments, but without addition of C_2H_2 (natural C_2H_4 production). Filtered seawater and coral fragments without addition of C_2H_2 showed negligible C_2H_4 production. C_2H_4 evolution rates of the biological samples were corrected for control signals (blank/biological ratios < 50%) and normalized to the surface area of the fragments. N_2 fixation rates are reported here as nmol C_2H_4 cm^{-2} h^{-1}. All coral fragments were returned to the maintenance aquarium for approximately one week before continuing with incubation measurements for organic matter release.

Organic matter release

OM release by the four coral genera was quantified during each of the four seasons using the established beaker incubation method[39,40,41]. Incubation chambers with a subset of the identical coral fragments (n=6, ca. 700 mL) and seawater controls (n=6, ca. 900 mL) were incubated for six hours during the day (10:00 – 16:00 h) in open chambers covered with transparent cellophane to prevent the input of airborne particles, leaving small side openings for air exchange. Chambers were not stirred to allow for comparisons with previous studies[40,41] and to rule out the influence of water currents on OM release and fractionation. Initial samples (50 mL) for DOC were immediately taken from the incubation water of each chamber at the start of the incubation with an acid-washed 50 mL polycarbonate syringe. Initial samples (1 L) for POC (n=3) were collected at the same time from the ambient aquarium water used to fill the chambers. At the end of the 6-hour incubation period, coral fragments were carefully removed from the incubation chambers with clean tweezers and 50 mL samples for DOC were immediately taken and processed using the same protocol as described for the *in situ* environmental monitoring. The remaining volume of incubation water (ca. 600 mL for coral chambers and ca. 800 mL for controls) was first measured and then vacuum-filtered onto pre-combusted GF/F filters for POC analyses following the protocol described above for *in situ* environmental monitoring. For the calculation of OM release rates (POC and DOC), concentration differences measured between the incubation start and end of each coral chamber were corrected by the average control signal and normalized by the filtered volume, incubation period and coral surface area. Total organic C (TOC) release was calculated as the sum of POC and DOC release for each coral fragment. OM release rates are reported here as μg C cm^{-2} h^{-1}.

Zooxanthellae density and chlorophyll a

For zooxanthellae density and chl *a* content analysis of the corals, a subset of the incubated coral fragments (n=4) was stored at -20°C prior to analysis in spring and in summer. Coral tissue was removed from the skeleton using a jet of pressurized air and 0.2 µm-filtered seawater. The resulting tissue slurry volume was brought to 50 mL with 0.2 µm-filtered seawater and homogenised using a vortex. One aliquot of 9 mL was subsequently subsampled, fixed in 3 mL 16% formaldehyde solution (final concentration 4%) and preserved at 4°C until zooxanthellae density analysis. Subsequently, the homogenate was centrifuged at 5000 rpm for 5 min, the supernatant removed and the pellet resuspended with a known volume of 0.2 µm-filtered seawater. The number of zooxanthellae was counted using an improved Neubauer haemocytometer[42]. The total number of zooxanthellae in the initial 50 mL slurry was calculated and normalized to coral surface area (cells cm^{-2}). Another aliquot of 5 mL was taken from each homogenate and immediately centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the pellet frozen at -20°C overnight to break the cells. The following day the pellet was resuspended in 10 mL 90% acetone and chl *a* was extracted for 24 h in the dark at 4°C. After another centrifugation run, chl *a* was measured using the non-acidification fluorometric method[43] on a Trilogy fluorometer equipped with the non-acidification chl module (CHL NA #046). Chl *a* content was normalized to coral surface area (µg chl *a* cm^{-2}) and to the total number of zooxanthellae of each fragment (pg chl *a* zooxanthella^{-1}).

Statistical analyses

To identify differences in Pn, R, Pg and N_2 fixation for the different coral genera during the four seasons we used a Two-way ANOVA design with the factor "Season" (fixed and orthogonal, 4 levels) and the factor "Genus" (fixed and orthogonal, 4 levels) and n=8. POC, DOC and TOC release rates were tested for differences with the same design but with n=6. Zooxanthellae density, areal chl *a* content and zooxanthellae chl *a* content were tested for differences with the same design but with the factor "Season" having 2 levels only (spring and summer) and n=4. Data were visually inspected for normality using q-q plots, tested for homogeneity of variances using the Cochran's C-test and transformed if necessary. In case of a significant interaction term, SNK (Student-Newman-Keuls) comparison tests were applied *a posteriori* to identify significant differences. To visualize multivariate changes in environmental variables among the four seasons, the unconstrained ordination of Principal Component Analysis

(PCA) was performed on previously normalized environmental data resulting from weekly averages. Differences in response to seasonality were tested using a PERMANOVA test[44]. The analysis was conducted using the Euclidean distance as coefficient of dissimilarity on previously normalized data. Type 3 (partial) sum of squares was used with unrestricted permutation of raw data (9999 permutations). All multivariate analyses were run using the PERMANOVA tool included in the Primer 6+ package. To determine whether the process of inorganic CO_2 fixation (Pg) was linked to the process of N_2 fixation in the corals over the four different seasons, linear models were fitted to the data and the significance of relationships was tested using the Pearson r-test in Statistica 12 software. CO_2 and N_2 fixation rates were established using proxies (O_2 for CO_2 and C_2H_4 for N_2). However, there is a direct relationship between these measures and the actual CO_2 and N_2 fixation rates[37,45,46], and the proxies chosen are routinely considered accurate surrogates for measurements of the latter rates in hard corals.

Results and Discussion

Our findings revealed that diazotrophs are consistently active and associated with all dominant hard coral genera (*Acropora*, *Pocillopora*, *Stylophora* and *Goniastrea*) throughout the year, despite the pronounced seasonality (Fig. 4.1). This association has previously been described in physiological and molecular studies[17, 18, 19, 20, 21, 22, 23], and N_2 fixation rates measured here are in the range of those reported for corals in the literature[8]. This highlights diazotrophs as constant members of hard coral holobionts. N_2 fixation significantly increased in all corals during summer (Fig. 4.1, Supplementary Table 4.S3, 4.S4). At the same time, gross photosynthesis did not show any consistent trend with regard to coral genus or season (Fig. 4.1). In summer, all corals exhibited a significant decrease in zooxanthellae density and areal chl *a* content, while a significant increase in zooxanthellae chl *a* content was detected (Fig. 4.2, Supplementary Table 4.S3 – 4.S6). The observed pattern of seasonal variability in zooxanthellae abundance (also known as physiological bleaching) represents an established phenomenon in tropical and subtropical scleractinian corals caused by an increase in photosynthetically active radiation, which enhances zooxanthellae loss presumably due to concomitant warmer temperatures[24, 25]. Moreover, growth efficiency and mitotic index of the symbiotic algae are temperature-dependent and decrease if temperature exceeds 25°C[26]. Despite the observed decreases in zooxanthellae population density, all corals maintained high gross productivity throughout the year (Fig. 4.1) indicating high metabolic plasticity to seasonal variation in environmental conditions.

Gross photosynthesis was positively and linearly correlated with N_2 fixation in summer, and to a minor extent in autumn (Fig. 4.3). The high sensitivity of nitrogenase (the enzyme responsible for N_2 fixation) to molecular and reactive oxygen species accumulating during photosynthesis requires a mechanism protecting nitrogenase from inactivation. The mechanism remains enigmatic, and may involve quenching via respiration and/or the Mehler reaction[17]. A possible explanation for the significant correlation between gross photosynthesis and N_2 fixation is that active diazotrophs provide the limiting N required for sustaining zooxanthellae photosynthesis in the nutrient-depleted waters of the Gulf of Aqaba, while obtaining energy in the form of C from the zooxanthellae within the coral host. Similar scenarios are well documented in land plants where heterotrophic diazotrophs in roots, such as rhizobia (also common partners in the coral holobiont[19, 20, 27]), fuel the plant with bioavailable N, while relying on plant photosynthesis to carry out the energetically demanding process of N_2 fixation. Unique to the coral system is that photosynthetic eukaryotic algae (zooxanthellae), prokaryotes (N_2 fixers) and the animal host (coral) cooperate, facilitating the nutritional success of the entire group of partners.

For tropical coral reefs, it is widely accepted that N is the most limiting nutrient to coral primary productivity[9]. Phosphorus (P) may also be limiting to coral metabolism[28], particularly in the case of an imbalanced nutrient supply with excess inorganic N (e.g., of anthropogenic origin)[29]. However, at our study site, the reef-surrounding seawater exhibits particulate organic C to N (POC:PN) and dissolved inorganic N to phosphate (DIN:PO_4^{3-}) ratios that are respectively higher and lower than the Redfield ratio (106:16:1) during all seasons (Supplementary Table 4.S1), indicating that N rather than P is the limiting nutrient to coral primary productivity. It is well-known that N limitation causes decreased pigment content in zooxanthellae[13], with N deficiency resulting in up to an 86% reduction in zooxanthellae chl *a* content after only one week[12]. Conversely, corals investigated here showed higher zooxanthellae chl *a* content (Fig. 4.2) and a positive linear relationship between N_2 and CO_2 fixation during the highly N-depleted summer period (Fig. 4.3). Overall, these findings suggest that microbial N_2 fixation is metabolically connected to photosynthetic algal CO_2 fixation and have important implications for our current understanding of the coral holobiont as a prominent example of a host-microbe ecosystem.

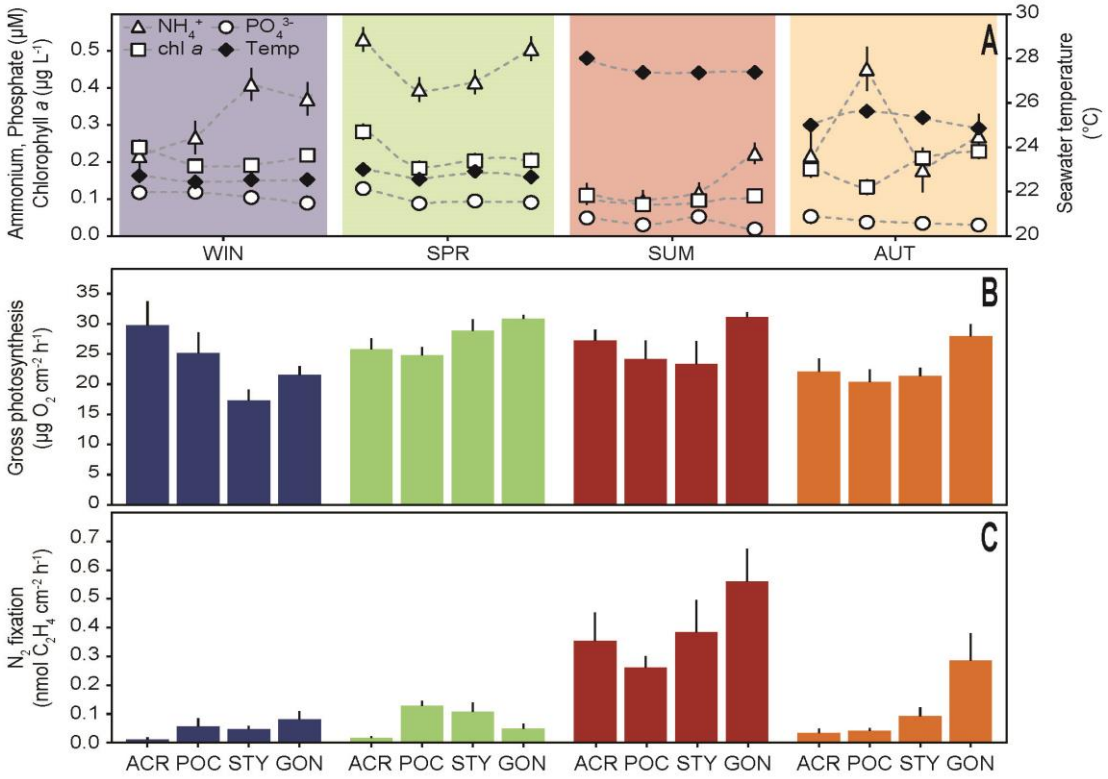

Figure 4.1. Seasonal environmental conditions with corresponding primary productivity and dinitrogen fixation in corals. (A) Seasonal weekly averages of the main environmental variables measured at the sampling location are shown. (B) Gross photosynthesis and (C) N_2 fixation of *Acropora* (ACR), *Pocillopora* (POC), *Stylophora* (STY) and *Goniastrea* (GON) are presented here as means (n=8) ± s.e.m.. See Supplementary Table 4.S1 – 4.S6 for the complete set of environmental variables and for statistical results. Colours represent winter (WIN, blue), spring (SPR, green), summer (SUM, red) and autumn (AUT, orange).

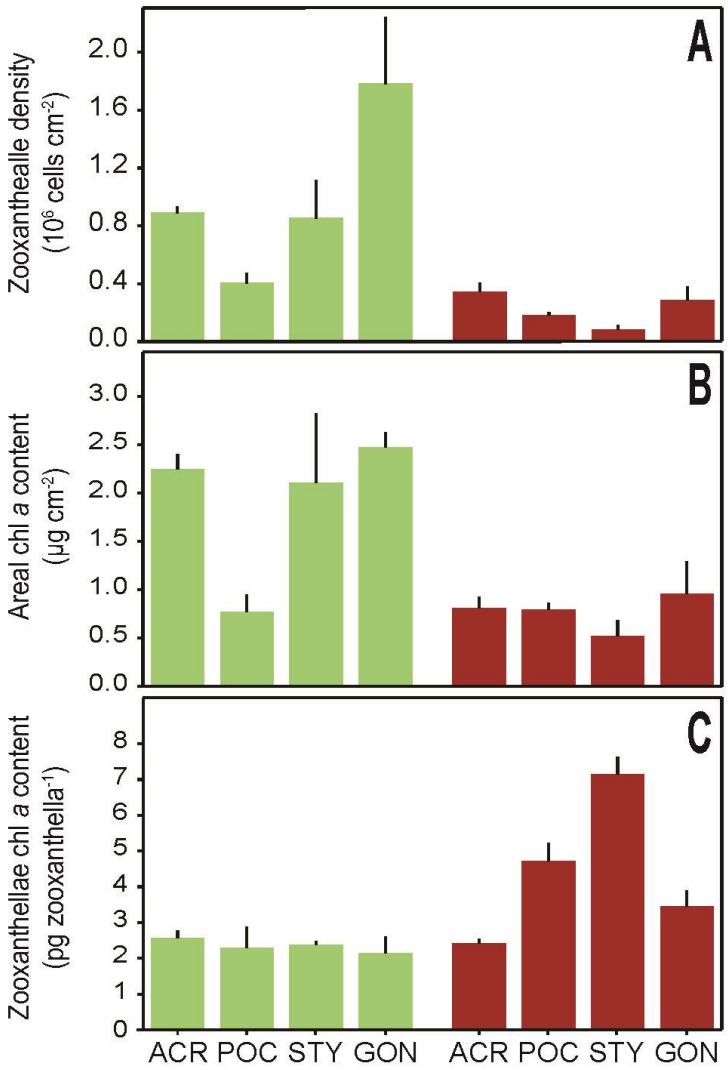

Figure 4.2. Seasonal zooxanthellae and photopigment content in corals. Shown are (A) zooxanthellae density, (B) areal chl a content, and (C) zooxanthellae chl a content. Colours represent spring (green) and summer (red). See Supplementary Data Table 4.S3 – 4.S6 for the statistical results. Data are presented as means (n=4) ± s.e.m..

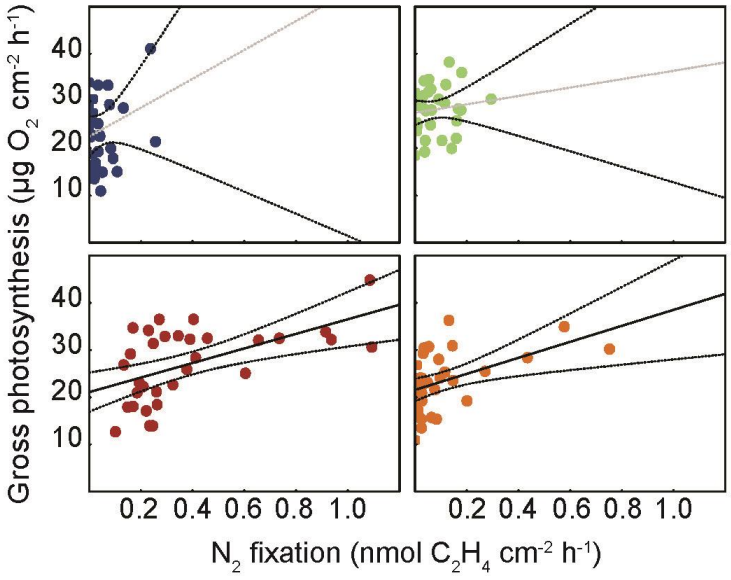

Figure 4.3. Seasonal relationships between N_2 fixation and gross photosynthesis in corals. Data points for each season (n=32) are colour coded in blue (winter), green (spring), red (summer) and orange (autumn). Best-fit linear regression lines (±95% confidence intervals) are solid black if the relationship is significant (winter: Pg=22.488+30.553x; r=0.205; p=0.261; spring: Pg=27.517+8.890x; r=0.128; p=0.484; summer: Pg=21.076+15.417x; r=0.551; p=0.001; autumn: Pg=21.565+16.940x; r=0.475; p=0.006).

C and N flux modelling (see supplementary information) was applied to investigate the relative importance of each N source to the zooxanthellae N demands (Fig. 4.4). In spring, when ambient DIN concentrations and uptake rates are high, but irradiance and water temperature are low, the coral host is replenished with N (Fig. 4.4, left panel) and actively limits algal population growth by removing excess nutrients from the intracellular milieu surrounding the zooxanthellae[11, 26]. Conversely, during the warm, high irradiance, nutrient-depleted summer (Fig. 4.4, right panel), environmental conditions trigger a dynamic expulsion of symbiotic algae leading to smaller zooxanthellae populations[24, 26]. However, N fixed and transferred by the thriving diazotrophic community to the zooxanthellae prevents N deficiency within the holobiont. Zooxanthellae are thus provided with sufficient N to sustain biosynthesis of the chlorin ring of chl *a*, the light-

capturing engine of photosynthesis. In summer, the contribution of N_2 fixation to zooxanthellae N demands (CZND) is comparable with the contribution of DIN uptake from reef-surrounding waters (Fig. 4.4, right panel). These two N sources together with heterotrophic N uptake facilitate 98 % of the total CZND, implying an almost complete N sufficiency of zooxanthellae (Fig. 4.4, Supplementary Table 4.S7).

These results importantly contribute to our understanding of coral metabolism; the dynamic equilibrium between the associated eukaryotic and prokaryotic communities in the coral holobiont (Fig. 4.4) sets the basis for high functional stability, sustaining high gross productivity despite changes in environmental conditions. Corals achieve this functional stability by adjusting population densities of symbiotic zooxanthellae and by benefiting from N fixed by symbiotic diazotrophs (Fig. 4.4). As a consequence of stable year-round primary productivity, hard corals are able to sustain high production and regeneration of their mucus surface layer and its concomitant release as organic matter (OM) (Fig. 4.4, Supplementary Fig. 4.S2). On a daily basis, we estimate that 10-12% of the total C and 14-28% of the total N acquired by the holobiont are lost via OM release (LOC and LON, respectively; Fig. 4.4). This released organic matter provides a food source for other reef organisms, thereby contributing to the functioning and productivity of the entire coral reef ecosystem[5, 6].

Recent research from various reef locations has identified diazotrophic assemblages in different coral species to be spatially and temporally consistent[19, 20, 27]. Concurrently, tropical reef systems worldwide typically exhibit extremely low seawater N concentrations, comparable to those measured in summer in the Gulf of Aqaba[30]. Further, no lower thresholds for nutrients considered limiting to coral reef growth have been found[30], implicating the widespread importance of N_2 fixation. Therefore, our results are contributing to the resolution of Darwin's paradox (by which coral reefs thrive in nutrient-depleted waters). We predict that the association with diazotrophs is fundamental to the functioning and high gross productivity of oligotrophic coral reefs worldwide and consequently to the essential goods and services (e.g., food and economic security, biodiversity[1]) that reef ecosystems provide.

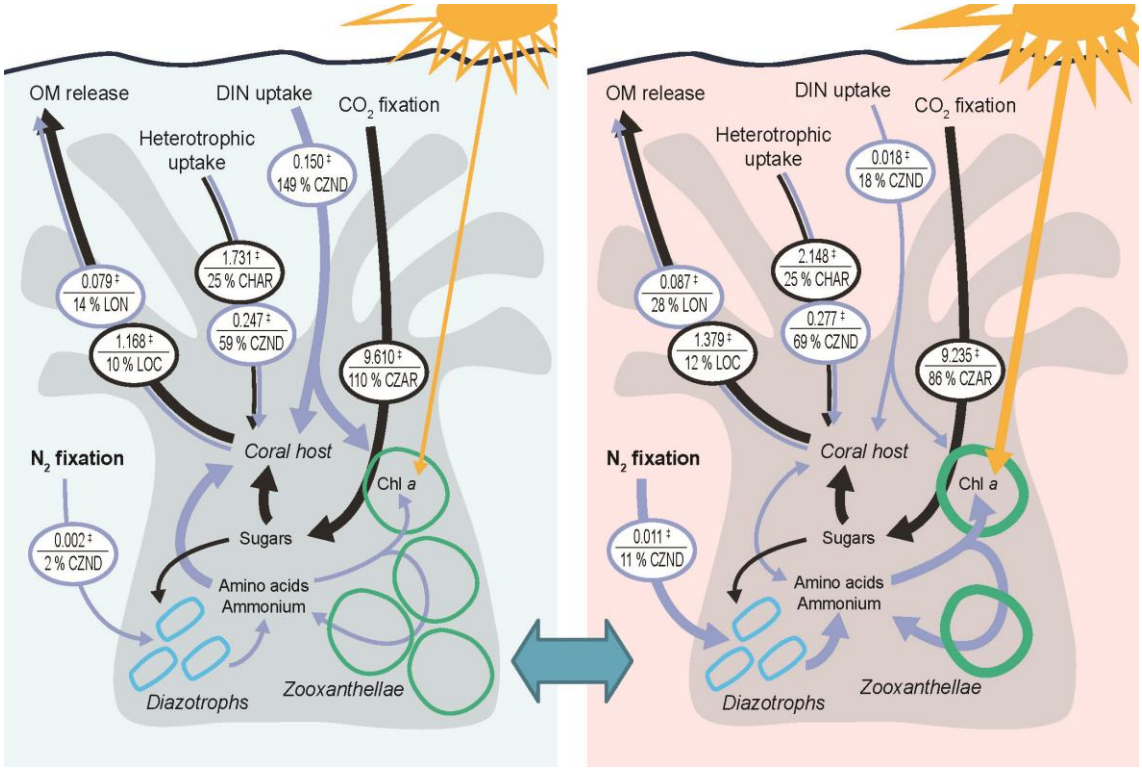

Figure 4.4. C and N flux model of the diazotroph-dinoflagellate-coral symbiosis. Shown are models for spring (left panel) and summer (right panel). Colours represent C (black) and N (blue) fluxes (‡ μmol cm^{-2} d^{-1}), and the width of arrows highlights seasonal differences. Percentages are: contribution of zooxanthellae-acquired N to zooxanthellae N demands (CZND); contribution of zooxanthellae-acquired C to animal respiration (CZAR); contribution of heterotrophically-acquired C to animal respiration (CHAR); loss by organic C release of the total acquired C (LOC); loss by organic N release of the total acquired N (LON); Parameters presented in the model (± standard deviation) and the respective calculations are reported online (supplementary information).

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Chapter V

Seasonal variation of N_2 fixation and O_2 fluxes associated with two dominant zooxanthellate soft corals from the Northern Red Sea*

Abstract

Dinitrogen (N_2) fixation by specialized prokaryotes (diazotrophs) represents an important source of bioavailable nitrogen (N) in the ocean. In coral reefs, several substrates and organisms are associated with diazotrophs, but potential N_2 fixation activity by zooxanthellate soft corals has not been investigated yet. Such soft corals may importantly contribute to the input of new N into the reef ecosystem as they can cover substantial benthic areas in today's coral reefs. Therefore, this study investigated N_2 fixation of two dominant zooxanthellate soft coral groups (*Sarcophyton* sp. and Xeniidae) in a Northern Red Sea fringing reef during all four seasons of one year. This was supplemented by respirometry incubations and *in-situ* monitoring of key environmental parameters. Findings revealed detectable N_2 fixation for both soft corals during all seasons. Annual N_2 fixation by *Sarcophyton* sp. was 3-times higher than that of Xeniidae, but both soft corals exhibited similar seasonal patterns. N_2 fixation significantly increased during summer, when water temperature and light intensity were highest, and inorganic nutrient availability was lowest. Coral respiration also peaked during summer and was positively correlated to N_2 fixation, while photosynthesis revealed maximum rates during the nutrient-enriched spring season. Given the importance of N for reproduction and growth, N_2 fixation may be a key component of soft coral nutrition during summer, when inorganic nutrient availability in the water column is lowest and likely not sufficient to sustain the high metabolic demand of soft corals.

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Introduction

Nitrogen (N) is a major limiting nutrient for primary productivity in oligotrophic coral reefs with dissolved inorganic nitrogen (DIN) concentrations often below 1µM (Hatcher 1990). Therefore, several reef organisms have evolved physiological mechanisms to conserve, recycle and collect the essential N (Szmant et al. 1990, Tanaka et al. 2006). Many symbiotic cnidarians (e.g. scleractinian corals) show a bidirectional translocation of N compounds between the zooxanthellae and the host. There, the zooxanthellae use the waste N compounds of their host (mostly in the form of ammonium), assimilate it into amino acids, and then translocate a portion of them back to the animal (Muscatine & Porter 1977, Rahav et al. 1989). In addition to this inner recycling loop, several benthic organisms (e.g. scleractinian corals, sponges) have evolved a mutualistic symbiosis with dinitrogen (N_2) fixing microbes (diazotrophs) that are able to convert atmospheric N_2 into bioavailable N (Fiore et al. 2010, Cardini et al. 2014). Up to 60 % of N fixed by diazotrophs can be released as dissolved organic N, thereby making it available for organisms unable to fix N_2 (Williams & Carpenter 1997). A recent study on scleractinian corals associated with diazotrophs demonstrated that the zooxanthellae are the primary users of the N_2 fixation products, implying that this process is important for coral photosynthesis and primary production (Lesser et al. 2007). Therefore, the ability of corals to overcome N limitation through N_2 fixation may determine their success in oligotrophic waters, ultimately influencing their ecological distribution and abundance on coral reefs (Fiore et al. 2010).

N_2 fixation in scleractinian corals has been described in several studies (Williams et al. 1987, Shashar et al. 1994a, Lesser et al. 2007), but there is a paucity of data available for soft corals, despite their common occurrence in tropical coral reef habitats (Benayahu & Loya 1981, Fabricius 1997). At present, N_2 fixation rates have been quantified for only two octocoral species (*Tubipora musica* and *Parerythropodium f. fulvum*), including one azooxanthellate soft coral (*Parerythropodium f. fulvum*; accepted name: *Rhytisma fulvum*) (Shashar et al. 1994b), while no data are available for zooxanthellate soft corals.

Some soft corals display opportunistic life-history features such as fast growth rates, high fecundity and asexual reproduction. These traits can give soft corals an ecological advantage compared to other benthic organisms that allows them to rapidly colonize large areas of a reef. Combined with environmental disturbance, this could result in benthic community shifts from hard to soft coral dominated reefs, which have been observed at several reef locations worldwide (Tilot et al. 2008, Norström et al. 2009). In the Northern Egyptian Red Sea, hard coral cover has declined by 5 - 25 % between 1996 and 2002, with a concurrent increase in zooxanthellate soft corals of the

families Xeniidae, Nephteidae and Alcyoniidae (Tilot et al. 2008). Thus, as soft corals in reefs of the Northern Red Sea are becoming an increasingly dominant benthic functional group they may contribute significantly to N_2 fixation an important biogeochemical process within coral reef functioning. . However, little is known about the capacity and contribution of soft corals to N_2 fixation within the reef and furthermore, on the relationship of their N_2 fixation with photosynthesis and environmental factors.

Several environmental factors including light, temperature, oxygen (O_2) concentrations and nutrient availability can affect marine N_2 fixation (Sohm et al. 2011, Knapp 2012, Cardini et al. 2014). However, thus far the effects of environmental variables on N_2 fixation have mostly been studied for free-living diazotrophs, while N_2 fixation associated with living corals has received much less attention. Lesser et al. (2007) found that N_2 fixation activity in the scleractinian coral *Monastraea cavernosa* follows a diurnal pattern with maximum rates during twilight and Davey et al. (2008) reported no effect of seasonal water temperature changes (22 vs 28 °C) on N_2 fixation associated with *Acropora aspera*. The coral reefs in the Gulf of Aqaba (Northern Red Sea) represent a natural laboratory for studying the effect of seasonally changing environmental conditions on coral physiology. Their high latitude location and the annual stratification cycle of the water column result in pronounced seasonal fluctuations in water temperature, light and inorganic nutrient availability (Silverman et al. 2007, Carlson et al. 2014).

In this study we thus investigated N_2 fixation rates associated with two of the most dominant zooxanthellate soft corals, Xeniidae and *Sarcophyton* sp. (family: Alcyoniidae), from a fringing reef of the Northern Gulf of Aqaba (Red Sea). Furthermore, responses of N_2 fixation, gross photosynthesis (P_{gross}) and dark respiration (R) rates (in terms of O_2) fluxes) to seasonally changing environmental conditions (e.g. inorganic nutrient concentrations, light intensity, water temperature) were studied over all four seasons within the year 2013 to identify how these two soft corals react to varying environmental conditions. Lastly, N_2 fixation was related to the P_{gross} and R rates of the corals in order to detect a potential linkage between diazotrophic activity and the physiology of the corals.

Methods

Description of study site

This study was carried out during two expeditions (January-April 2013 and August-December 2013) to the Marine Science Station (MSS) at the Northern Gulf of Aqaba, Jordan (29° 27`N, 34° 58`E). The MSS is situated at the Jordanian Red Sea coast approximately 10 km south of Aqaba city with access to a fringing coral reef inside a marine reserve. In order to study the effect of seasonally changing environmental conditions on soft coral physiology, all experiments described below were conducted once in each of the months to represent all four seasons: February (Winter), April (Spring), September (Summer), and November (Fall) during the year 2013.

Soft coral distribution

Line point intercept (LPI) transects were carried out to determine relative soft coral cover in the study area. Three replicate 50 m LPI transects were conducted during each season at 1, 5, 10 and 20 m water depth. The benthic cover was recorded at 0.5 m intervals directly below the transect line (101 recorded data points per LPI transect). The relative abundance of soft corals was calculated as their percentage of benthic coverage.

Table 5.1. Summary of the environmental water parameters monitored over four weeks during each of the four different seasons at 10 m water depth. During each season, water temperature was continuously recorded and is averaged over the four weeks period, while light intensity represents maximum values measured daily between 11:00 and 13:00. Ammonium and phosphate concentrations were measured once a week from seawater samples (n = 4) taken in the early morning. Values are represented as means with SE in parentheses.

Month (season)	Temperature (°C)	Light intensity (PAR)	Ammonium (µM)	Phosphate (µM)
Jan/Feb (winter)	22.97 (0.11)	180 (15)	0.32 (0.04)	0.11 (0.01)
Mar/Apr (spring)	22.78 (0.10)	257 (9)	0.46 (0.03)	0.10 (0.01)
Sep (summer)	27.52 (0.16)	317 (17)	0.14 (0.03)	0.04 (0.01)
Nov (fall)	25.19 (0.17)	159 (18)	0.28 (0.06)	0.04 (0.01)

Table 5.2. Benthic soft coral cover in the different water depths and the relative contribution by *Sarcophyton* sp., Xeniidae and other soft corals. Values are represented as annual means (n = 12) with SE in parentheses.

	Benthic cover (%)	**Soft coral composition (%)**		
Depth (m)	Soft corals	*Sarcophyton* sp.	Xeniidae	Others
1	3.2 (0.6)	10.9 (8.7)	2.3 (2.2)	86.8 (8.7)
5	3.0 (0.7)	3.2 (1.9)	57.4 (12.1)	45.4 (11.9)
10	21.6 (1.3)	5.4 (2.3)	91.0 (2.2)	3.5 (1.3)
20	6.6 (0.6)	6.7 (2.7	88.8 (3.5)	4.5 (2.3)
Average	8.6 (0.8)	6.6 (3.9)	59.9 (5.0)	35.1 (6.1)

Soft coral collection and maintenance

Individual colonies of the genus *Sarcophyton* sp. (n = 8, average polyp number = 459 ± 41) and the family Xeniidae (n = 8, average polyp number = 64 ± 4) were collected during each season from the reef slope at 10 m water depth using SCUBA. To prevent any tissue damage, all soft corals were collected along with a small piece of the anchoring rock (< 0.5 cm diameter) which they were attached to using hammer and chisel. Subsequently, individual coral colonies were fixed, with their attachment rock, onto ceramic tiles using a two-part epoxy putty (Reef Construct, AQUA MEDIC GmbH, Bissendorf, Germany). The putty was used to cover the anchoring rock to ensure that encrusting communities on the rock did not affect rate measurements during following incubations. All corals were transferred to an outdoor 800 L flow-through aquarium supplied with seawater pumped directly from the reef at 10 m water depth (exchange rate: 4000 L h^{-1}), thereby providing *in-situ* water temperature and nutrient levels. Layers of netting were positioned above the tank to adjust light levels to those measured *in-situ* at 10 m water depth with HOBO loggers (Onset HOBO Pendant UA-002-64; spectral detection range: 150 – 1200 nm; temperature accuracy: ± 0.53 °C; Bourne, MA, USA). The corals were allowed to acclimate for 1 week before further experimentation. All incubations took place in the outdoor 800 L flow-through aquarium to ensure the same water temperature, nutrient and light conditions and to avoid any stress to the coral colonies.

Quantification of O_2 fluxes

The tiles with the attached corals were carefully cleaned of algal turf using a tooth brush. Each coral colony (n = 8) was transferred, without exposure to air, to individual 1 L glass chambers. In addition, 8 chambers filled only with seawater served as controls to measure planktonic background metabolism. The start O_2 concentration in each chamber was measured using a salinity corrected O_2 optode sensor (FDO®925 – Optical Dissolved Oxygen Sensor, range: 0.00 - 20.00 mg O_2 L^{-1}, accuracy: ± 0.5% of the value, MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). All chambers were sealed gas-tight (without any air bubble inside) and incubated twice with constant stirring (600 rpm) on magnetic stirring plates for 1-2 h, respectively (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific™). After that, each chamber was opened in order to measure the end O_2 concentrations. The first incubation was carried out 1-2 h after sunset to measure R in complete darkness, while the second one started the following day between 12:00 and 13:00, for net photosynthesis (P_{net}) determination. To calculate O_2 fluxes (P_{net} and R) from dark and light incubations, O_2 start concentrations were subtracted from end concentrations and the results were normalized by incubation time. Finally, O_2 fluxes were corrected for the seawater control signal, related to incubation volume and normalized to the coral surface area. P_{gross} rates were subsequently calculated according to $P_{gross} = P_{net} + R$. As P_{net} was measured during highest daily irradiance levels and dark R rates were shown to be significantly lower than light R rates for corals during active photosynthesis (Fabricius & Klumpp 1995, Al-Horani et al. 2003), the presented P_{gross} rates are conservative estimates of the daily maximum O_2 production.

Quantification of N_2 fixation

N_2 fixation rates of the same soft coral colonies were measured 3-4 h after the light incubation for quantification of P_{net} ended. An adapted acetylene (C_2H_2) reduction assay was applied as it was recently confirmed to be applicable for N_2 fixation quantification in oligotrophic waters (Capone 1993, Wilson et al. 2012). C_2H_2 gas was freshly generated from calcium carbide and bubbled through seawater in order to produce C_2H_2-enriched seawater. Without air exposure, each coral colony (n = 8) was placed individually in a 1 L glass chamber containing 800 ml unfiltered seawater and 200 ml of air headspace. Then, 10 % of the seawater (80 ml) was replaced with C_2H_2-enriched seawater before the chambers were closed gastight. Immediately after, 10 % of the headspace (20 ml) was replaced by C_2H_2 gas. The addition of C_2H_2 to the seawater minimizes the

lag phase of the ARA due to a faster equilibration of C_2H_2 between the gas and liquid phase and an immediate C_2H_2 saturation of the nitrogenase enzyme. In addition, 4 sets of controls were also tested for ethylene (C_2H_4) production: 1. Unfiltered seawater control (without coral fragments, n = 8); 2. 0.2 μm-filtered seawater control (without coral fragments, n = 6); 3. Tiles (without coral fragments) in unfiltered seawater (n = 6); 4. Coral fragments in unfiltered seawater without C_2H_2 addition (natural C_2H_4 production, n = 6). Over the whole incubation period (24 h), all chambers were constantly stirred (600 rpm), and gas samples were taken at the incubation start and after 4, 12, 16 and 24 h. At each of these time intervals, 1 ml of gas sample was collected with a gastight syringe from each chamber, transferred into gastight 2 ml vials previously filled with distilled water, and stored frozen upside down until analysis. C_2H_4 concentrations in the gas samples were measured in the field laboratory using a customized reducing compound photometer (RCP) (Peak Laboratories) with a detection limit of 100 ppb. The higher sensitivity of the RCP compared to the commonly used GC-FID (gas chromatograph equipped with a flame ionization detector) allows best accurate estimations of C_2H_4 reduction rates. Calibration of the RCP was conducted using serial dilutions of a 200 ± 4 ppm C_2H_4 standard in air (Restek, Bellefonte, PA, USA). To calculate C_2H_4 production rates of the coral fragments C_2H_4 evolution rates of the biological samples were corrected for seawater control signals (blank/biological ratios: 0.15 – 0.57) and subsequently normalized to incubation time and coral surface area. All rates are reported as nmol C_2H_4 cm^{-2} h^{-1} since no parallel ^{15}N incubations were conducted, and the use of a theoretical conversion factor is controversial as the ^{15}N method may have largely underestimated N_2 fixation until recently (Mohr et al. 2010). Additionally, it allows best comparability with the current literature as the C_2H_2 reduction method has been most widely applied for benthic N_2 fixation quantification measurements in coral reefs (Table 5.6).

Surface area determination of soft corals

Activity rates (N_2 fixation, P_{gross} and R) for each colony were related to coral surface area that was quantified using an advanced geometry approach (Naumann et al. 2009). This approach has already been applied for soft corals in Bednarz et al. (2012) and has been commonly used in physiological coral reef studies thereby allowing best comparability of the present data to previous studies. Briefly, the number of polyps of each coral colony was counted, and each of these polyps was mathematically handled as the area of a circle. The surface area of 50 completely expanded polyps randomly distributed over all colonies was measured separately for Xeniidae and *Sarcophyton* sp. using the image analysis software ImageJ (National Institutes of Health, USA). Subsequently,

the average circular surface area of a polyp was calculated ($r^2 * \pi$) and multiplied by the number of polyps per colony. In addition, the surface area of the body foot of each colony was approximated to a cylinder ($2 * \pi * r * h$) by measuring its diameter ($2 * r$) and height (h) using a caliper (accuracy ± 0.01 cm). To generate the total surface area of each incubated Xeniidae and *Sarcophyton* sp. colony, the total number of polyps of each colony was multiplied with the average surface area of an expanded polyp and subsequently added to the surface area of the body foot.

Monitoring of environmental parameters

During each season water temperature and light intensity (lux) at 10 m water depth were continuously recorded over four weeks by data loggers (Onset HOBO Pendant UA-002-64; temperature accuracy: ± 0.53 °C, spectral detection range: 150 – 1200 nm; Bourne, MA, USA). The presented light intensities are standardized to the time of day with maximum light intensities (11:00 - 13:00) and lux readings were converted to photosynthetically active radiation (PAR, µmol quanta m^{-2} s^{-1}, wavelength 400 to 700 nm) using the following approximation: 1 µmol quanta m^{-2} s^{-1} = 52.0 lux. This conversion factor was obtained by inter-calibrating the lux readings with data obtained from a PAR sensor (LI-COR LI-192SA underwater quantum sensor) during a simultaneous minute-by-minute measurement over 5 h. Both readings correlated well (r^2 = 0.83) and the obtained conversion factor of 52.0 was very similar to 51.2 reported by Valiela (1984). Once a week, seawater samples (50 ml, n = 4) were taken from the place of coral collection. After filtering the seawater through sample-washed cellulose acetate membrane filters (nominal pore size: 0.45 µm), inorganic nutrient (ammonium, phosphate) concentrations were immediately measured following methods described by Holmes et al. (1999) and Murphy & Riley (1962). Ammonium was determined fluorimetrically using a Trilogy Fluorometer (Turner Designs) with a detection limit of 0.09 µM, while phosphate was measured photometrically with a JASCO-V630 spectrophotometer and a detection limit of 0.01 µM.

Statistical analysis

All statistical analyses were carried out using Primer-E version 6 software (Clarke & Gorley 2006) with the PERMANOVA+ add on (Anderson 2001). Analyses were based on Euclidean distance of environmental data (normalized) and on Bray Curtis similarities of physiological parameters (square-root transformed). A principal coordinate analysis and a one-factor

PERMANOVA with type III (partial) sum of squares and unrestricted permutation of raw data (999 permutations) was used to test for seasonal differences of the environmental variables (water temperature, light intensity, inorganic nutrients = co-linear variables ammonium and phosphate are summarized). Two-factor PERMANOVAs were performed to test for differences of physiological parameters (N_2 fixation, P_{gross}, R) between soft corals and seasons. Therefore, type I (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations), and pairwise-tests were carried out when significant differences occurred. In addition, a non-metric multidimensional scaling ordination of the physiological parameters (N_2 fixation, P_{gross}, and R) in relation to soft coral and season was conducted. Finally, correlations between the physiological variables as well as between the physiological variables and the environmental factors were determined *via* linear regression.

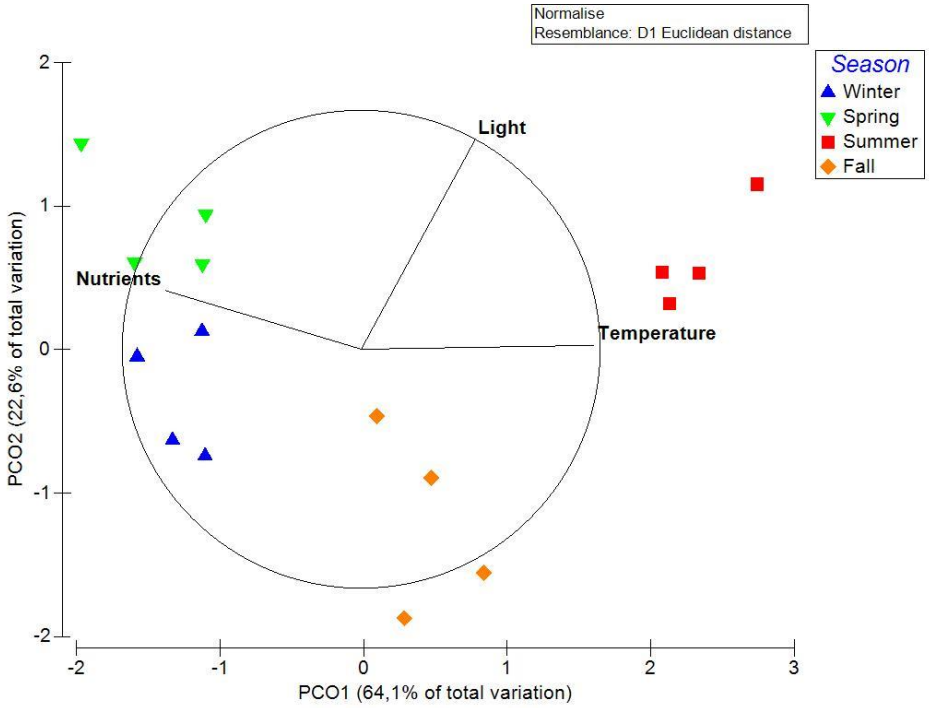

Figure 5.1. Principle coordinates analysis performed on Euclidian distance matrix and normalized data for the different environmental variables (temperature, light, inorganic nutrients) measured in seawater samples from 10 m water depth once a week during four different seasons (winter, spring, summer, fall).

Results

Environmental conditions and soft coral cover

The four seasons differed significantly from each other with respect to the environmental water parameters (PERMANOVA, *df*=3, SS=51.138, MS=17.046, Pseudo-F=23.083, *p*=0.001). The winter and spring season were most similar to each other, followed by fall with lower inorganic nutrient concentrations and higher water temperatures. The summer season was most distinct to the other three seasons, and exhibited the highest water temperatures and light intensities, but the lowest inorganic nutrient concentrations (Fig. 5.1; Table 5.1).

Despite these strong seasonal changes in environmental water parameters, the soft coral cover remained constant throughout the year at each water depth (Table 5.2). Over all depths, soft coral cover was approximately 8.6 ± 0.8 %, whereby the highest cover was recorded at 10 m water depth with a percentage cover of 21.6 ± 1.3 %. Most of the soft coral cover was represented by Xeniidae that was approximately 10 times more abundant than *Sarcophyton* sp.

N_2 fixation and O_2 fluxes

Active N_2 fixation rates occurred throughout the year for both soft corals indicated by the up to 75-fold higher C_2H_4 production in coral containing incubation chambers compared to the seawater controls (Table 5.3). Soft coral-associated N_2 fixation revealed significant effects of soft coral, season and their interaction (Fig. 5.2; Table 5.4). The corals exhibited similar rates during the winter season, but *Sarcophyton* sp. showed significantly higher rates in the other three seasons compared to Xeniidae. Rates ranged during the year from 0.004 to 0.205 nmol C_2H_4 cm^{-2} $h^{-1}$for *Sarcophyton* sp. and from 0.001 to 0.096 nmol C_2H_4 cm^{-2} $h^{-1}$for Xeniidae with annual averages of 0.055 ± 0.011 nmol C_2H_4 cm^{-2} h^{-1} and 0.019 ± 0.005 nmol N_2 cm^{-2} h^{-1}, respectively. Besides these soft coral-specific differences, both soft corals showed the same seasonal pattern of N_2 fixation activity. While no differences occurred between winter, spring and fall, N_2 fixation rates significantly increased during the summer season for both investigated soft corals. Overall, summer rates were 3-6 (*Sarcophyton* sp.) and 6-14 times (Xeniidae) higher than N_2 fixation during the other seasons.

O_2 fluxes (P_{gross} and R) also exhibited soft coral-specific and seasonal differences (Fig. 5.2, Table 5.4). Averaged among all seasons, Xeniidae (15.6 ± 0.8 μg O_2 cm^{-2} h^{-1}) revealed significantly

higher P_{gross} rates, approximately 1.5-fold higher than *Sarcophyton* sp. (11.7 ± 0.8 μg O_2 cm^{-2} h^{-1}). On a seasonal scale, Xeniidae displayed the highest P_{gross} rates during spring, while *Sarcophyton* sp. had maximum rates both during spring and summer. In contrast, R rates were constantly lower in Xeniidae (2.8 ± 0.2 μg O_2 cm^{-2} h^{-1}) compared to *Sarcophyton* sp. (4.9 ± 0.3 μg O_2 cm^{-2} h^{-1}) averaged among all seasons. Xeniidae exhibited highest R rates both in spring and summer, while R rates of *Sarcophyton* sp. peaked during the summer season, thereby following the seasonal pattern of N_2 fixation rates.

Table 5.3. N_2 fixation (nmol C_2H_4 L^{-1} h^{-1}) measured in soft coral and seawater control (without corals) incubation chambers. Values are normalized to 1 L incubation water and presented as mean (SE) of n = 8 replicates.

Incubation	Winter	Spring	Summer	Fall
Sarcophyton sp.	2.07 (0.83)	4.05 (0.92)	14.26 (1.77)	3.57 (1.47)
Xeniidae	1.20 (0.25)	0.68 (0.08)	2.13 (0.57)	0.61 (0.12)
Seawater control	0.06 (0.04)	0.36 (0.05)	0.19 (0.06)	0.15 (0.08)

Table 5.4. Results of two-factorial PERMANOVAs for N_2 fixation, P_{gross} and R rates for the two soft corals (*Sarcophyton* sp. and Xeniidae) and the four different seasons (winter, spring, summer, fall). Soft coral and season were fixed effects. PERMANOVA was based on Bray Curtis similarity after square root transformation. Type 1 (sequential) sum of squares was used with permutation of residuals under a reduced model (999 permutations). Significant *p* values are in bold.

Variables	Effect	*df*	SS	MS	Pseudo *F*	*p* value
N_2 fixation	Soft coral (Sc)	1	10144	10144	31.34	**0.001**
(nmol C_2H_4 cm^{-2} h^{-1})	Season (Se)	3	19555	6519	20.14	**0.001**
	Sc x Se	3	5208	1736	5.36	**0.001**
P_{gross}	Soft coral (Sc)	1	918	918	16.66	**0.002**
(μg O_2 cm^{-2} h^{-1})	Season (Se)	3	1350	450	8.17	**0.001**
	Sc x Se	3	129	43	0.78	0.513
R	Soft coral (Sc)	1	2534	2534	77.52	**0.001**
(μg O_2 cm^{-2} h^{-1})	Season (Se)	3	1503	501	15.33	**0.001**
	Sc x Se	3	332	111	3.38	**0.032**

Relationships between N_2 fixation, O_2 fluxes, and environmental factors

N_2 fixation and O_2 fluxes (P_{gross} and R) clearly showed a separation between the two soft corals as well as a distinct difference in summer compared to the other three seasons (Fig. 5.3). Separation of summer from the other seasons was mainly driven by changes in N_2 fixation and R rather than by changes in P_{gross}, suggesting a potential linkage between N_2 fixation and R. Indeed, linear regression analysis revealed a significant positive relationship between R and N_2 fixation for *Sarcophyton* sp. (F = 16.070, r^2 = 0.373, p< 0.001), but not for Xeniidae (F = 1.249, r^2 = 0.043, p = 0.273). In contrast, no significant relationship between P_{gross} and N_2 fixation was found, neither for *Sarcophyton* sp. (F = 2.784, r^2 = 0.093, p = 0.107) nor for Xeniidae (F = 0.047, r^2 = 0.002, p = 0.8297).

N_2 fixation of both soft corals showed similar relationships with each water parameter, with negative correlations to ammonium and phosphate concentrations and positive correlations to water temperature and light intensity (Table 5.5). R rates of *Sarcophyton* sp. showed the same correlation to the water parameters as N_2 fixation. In contrast, no correlations between R rates of Xeniidae and any water parameters were found. P_{gross} of Xeniidae was positively correlated to ammonium concentration, while P_{gross} of *Sarcophyton* sp. was positively correlated to light intensity.

Table 5.5. Linear regression analysis (r^2 values) between N_2 fixation, P_{gross}, R rates of the two soft corals (*Sarcophyton* sp. and Xeniidae) and the four different environmental water parameters (ammonium concentration, phosphate concentration, water temperature and light intensity). Bold characters indicate significant positive relationships and italicized characters indicate significant negative relationships (* p<0.05, ** p<0.005, *** p<0.001).

	Ammonium	Phosphate	Water temperature	Light intensity
Sarcophyton sp.				
N_2 fixation (nmol C_2H_4 cm^{-2} h^{-1})	*0.217**	*0.137**	**0.344*** **	**0.478*** **
P_{gross} (µg O_2 cm^{-2} h^{-1})	0.001	0.047	0.060	**0.180***
R (µg O_2 cm^{-2} h^{-1})	*0.142**	*0.158**	**0.326****	
Xeniidae				
N_2 fixation (nmol C_2H_4 cm^{-2} h^{-1})	*0.561****	*0.222***	**0.592*** **	**0.513*** **
P_{gross} (µg O_2 cm^{-2} h^{-1})	**0.144***	0.022	0.021	0.130
R (µg O_2 cm^{-2} h^{-1})	0.040	0.000	0.002	

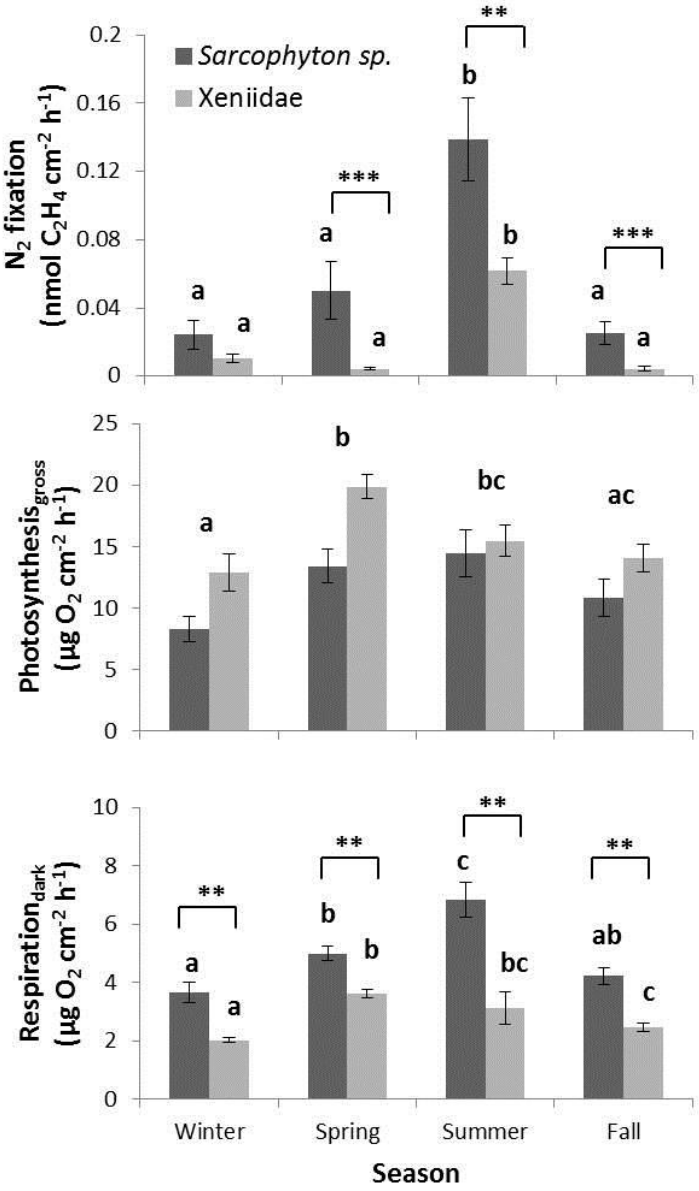

Figure 5.2. Rates of N_2 fixation, P_{gross} and R for *Sarcophyton* sp. and Xeniidae measured during four different seasons (winter, spring, summer, fall) during 2013. Values are given as mean ± SE (n=8). Asteriks indicate significant differences between the two soft corals during each season (* $p<0.05$, ** $p<0.005$, *** $p< 0.001$) and different letters indicate significant differences between the seasons for *Sarcophyton* sp. and Xeniidae, respectively, based on pair-wise PERMANOVA analysis.

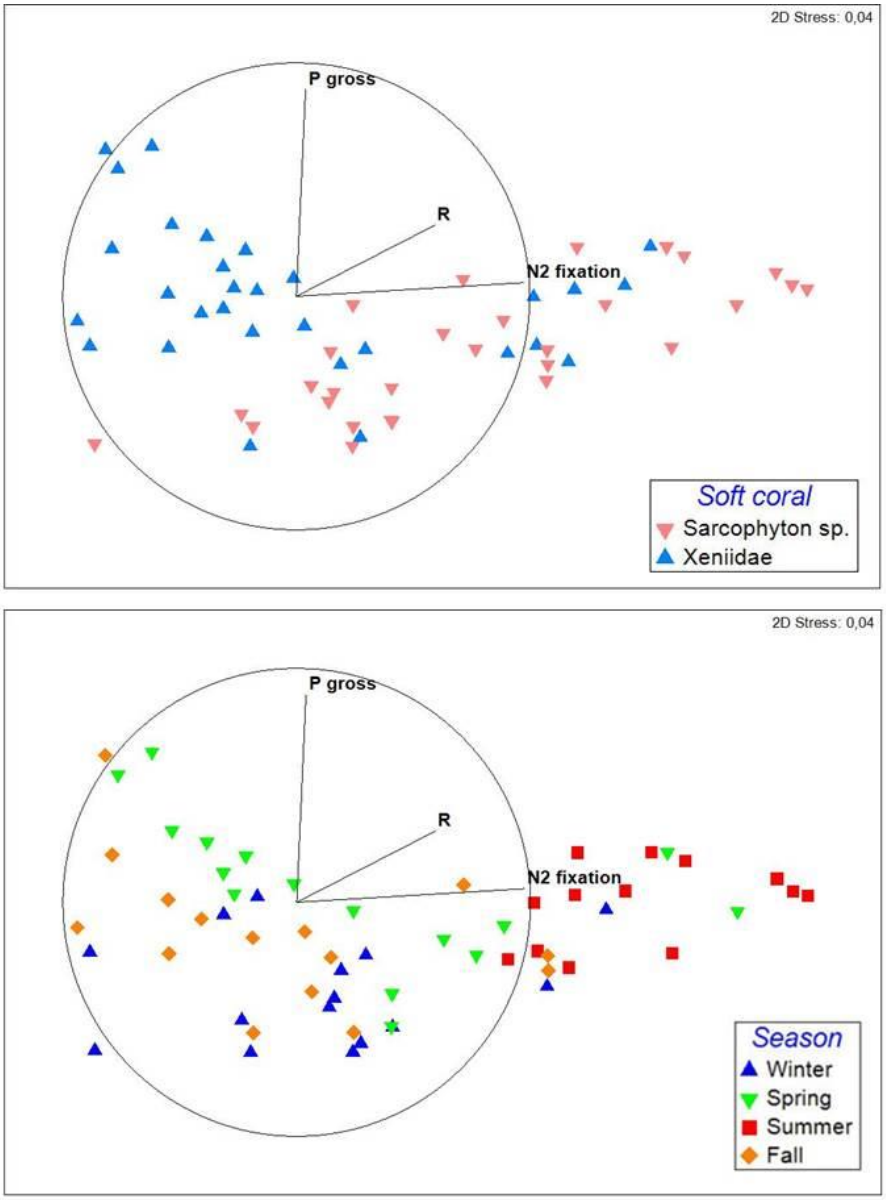

Figure 5.3. Multidimensional scaling plot of N_2 fixation, P_{gross} and R rates for the two soft corals (*Sarcophyton* sp. and Xeniidae) and the four different seasons (winter, spring, summer, fall). Analysis was performed on Bray Curtis similarities of square root transformed data.

Discussion

Soft coral-specific N_2 fixation and O_2 fluxes

This study for the first time identified N_2 fixation rates associated with two dominant zooxanthellate soft corals from an oligotrophic, subtropical coral reef environment. Previous studies, mainly focused on hard corals, identified their different hard coral-associated N_2 fixing bacteria (Rohwer et al. 2002, Wegley et al. 2007, Olson et al. 2009, Lema et al. 2012), or quantified their N_2 fixation rates (Shashar et al. 1994a; Davey et al. 2008; Lesser et al. 2007). These rates are 1-2 orders of magnitude higher than the rates detected for *Sarcophyton* sp. and Xeniidae in the present study (Table 5.6). These apparent differences in N_2 fixation rates may be due to abundance and composition differences of the diazotrophic communities associated with hard and soft corals. Lema et al. (2012) demonstrated that hard coral species form specific associations with diazotrophs which may further result in species-specific N_2 fixing activities. Likely, hard and soft corals may harbor a very distinct diazotrophic community due to the presence of endolithic algae and endolithic bacteria in the former. Endolithic, heterotrophic bacteria are most likely responsible for the majority of N_2 fixation activity in scleractinian corals (Shashar et al. 1994a), which was supported by measurements of endolithic ammonium excretion rates (0.8 - 1.4 nmol NH_4^+ cm^{-2} h^{-1}) that matched the rates of N_2 fixation (Ferrer & Szmant 1988). The main energy source for N_2 fixation is likely provided *via* the excretion of organic photosynthates by the coral into the coralline skeleton, thereby establishing a suitable microhabitat for a diazotrophic community that is absent in soft corals. Therefore, soft corals may harbor less diazotrophs compared to scleractinian corals that may explain their lower N_2 fixation rates.

So far, N_2 fixation has only been investigated for two octocoral species (*Tubipora musica* and*Rhytisma fulvum*) using the acetylene reduction assay (Shashar et al. 1994b). They measured rates of 35.7 ± 14.2 nmol C_2H_4 cm^{-2} h^{-1}, thus exceeding rates quantified for *Sarcophyton* sp. and Xeniidae by 2-3 orders of magnitude. The octocoral species *T. musica* contains an endoskeleton providing habitat for endolithic diazotrophs comparable to scleractinian corals, while *R. fulvum* is an azooxanthellate, encrusting soft coral species. Because of its encrusting morphology, *R. fulvum* is extremely difficult to separate from its attaching substrate and Shashar et al. (1994b) did not clean the soft coral from epilithic algae. This may result in biased N_2 fixation rates as also the activity of diazotrophs which are not associated with the coral is taken into account. Shashar et al. (1994b) also present N_2 fixation rates of other unidentified cnidarians and sponges, where the rates from the present study are well within. Overall, this demonstrates that there is a wide range of N_2 fixation activity among different benthic coral reef organisms.

Although N_2 fixation rates associated with *Sarcophyton* sp. and Xeniidae are in the lower range among benthic reef organisms, the rates are up to 75-fold higher compared to N_2 fixation in the seawater controls, thus confirming that the two soft corals are associated with active N_2 fixing bacteria. N_2 fixation in the seawater controls (0.8 to 4.3 nmol N L^{-1} d^{-1}, obtained using 4:1 as C_2H_4:N_2 conversion factor) is slightly higher but within the range of seawater N_2 fixation previously measured in the Gulf of Aqaba using the $^{15}N_2$ method (0.1 to 1.9 nmol N L^{-1} d^{-1}; Foster et al. 2009). The classical $^{15}N_2$ method underestimates N_2 fixation rates (Mohr et al. 2010) when the $^{15}N_2$ tracer is only introduced as a gas bubble as in Foster et al. (2009), while the addition of $^{15}N_2$ enriched seawater accelerates the gas equilibration process and improves the accuracy of the method (Wilson et al. 2012). Similarly, the acetylene reduction method can reliably quantify N_2 fixation in oligotrophic waters if acetylene-enriched seawater is used as in the present study (Wilson et al. 2012). These methodological differences provide reasoning for the slightly higher rates obtained in our seawater controls and confirm the validity of the soft coral-associated N_2 fixation rates.

The two zooxanthellate soft corals investigated in the present study also revealed differences in their specific N_2 fixation activity with significantly higher rates in *Sarcophyton* sp. compared to Xeniidae on an annual average. Lower N_2 fixation rates in Xeniidae may be caused by high oxygenated areas due to higher P_{gross} and lower R rates, which indicate a more autotrophic nutrition by Xeniidae compared to *Sarcophyton* sp. This is confirmed by the study of Schlichter et al. (1983) that characterized soft corals of the family Xeniidae as functional autotrophic plant animals. Also, compared to *Sarcophyton* sp., Xeniidae exhibit non-retractile, pumping polyps thereby creating conditions which facilitate photosynthesis. Pumping increases water exchange between the boundary layer of the organism and the water body (Mass et al. 2010, Kremien et al. 2013), while polyp expansion increases the surface area for potential gas exchange through the epidermal tissue (Fabricius & Klumpp 1995). Furthermore, the polyp's tip hosts most of the organism's zooxanthellae and represents the photosynthetically most active tissue. Polyp retraction reduces light exposure for zooxanthellae, and can decrease photosynthesis in several soft corals (Fabricius & Klumpp 1995). This may help explain the higher photosynthesis rates measured in Xeniidae during each season, which likely leads to hyperoxic conditions in the tissue during daytime (Shashar et al. 1993, Kühl et al. 1995). As the nitrogenase enzyme is highly sensitive to O_2 (Postgate 1982) it may explain lower N_2 fixation rates in Xeniidae compared to *Sarcophyton* sp.

Table 5.6. Reported N_2 fixation rates (nmol C_2H_4 cm^{-2} h^{-1}) for benthic reef organisms and substrates in comparison with annual averages of the soft corals *Sarcophyton* sp. and Xeniidae from the present study.

Organism/Substrate	N_2 fixation	Location	Reference
Octocorals			
Xeniidae	0.001-0.096	Aqaba, Red Sea	This study
Sarcophyton sp.	0.004-0.205	Aqaba, Red Sea	This study
T. musica and *R. fulvum*	35.7 ± 14.2	Eilat, Red Sea	Shashar et al. 1994b
Scleractinian corals			
Acropora aspera	0.56-1.16	Australia, GBR	Davey et al. 2008
Acropora sp.	8.7 ± 7.3	Eilat, Red Sea	Shashar et al. 1994a
Stylophora pistillata	6.4 ± 2.4	Eilat, Red Sea	Shashar et al. 1994a
Pocillopora damicornis	0.6 ± 0.4	Eilat, Red Sea	Shashar et al. 1994a
Other cnidarians	0.1 ± 0.3	Eilat, Red Sea	Shashar et al. 1994b
Sponges	0.2 ± 0.4	Eilat, Red Sea	Shashar et al. 1994b
Dead coral skeleton	55.45 ± 28.5	Eilat, Red Sea	Shashar et al. 1994b
Dead coral skeleton	0.15-12.77	Australia, GBR	Davey et al. 2008
Algal substrate	9.25 ± 0.5	Eilat, Red Sea	Shashar et al. 1994b
Microbial mats	0.59-2.97	Indian Ocean	Charpy et al. 2012
Carbonate sand	0.18 ± 1.02	French Polynesia	Charpy-Roubaud et al. 2001
Carbonate sand	19.5 ± 17.5	Eilat, Red Sea	Shashar et al. 1994b

Seasonal variation in N_2 fixation and O_2 fluxes

In the present study, we measured for the first time the effect of seasonally changing environmental conditions on N_2 fixation associated with soft corals. Both of the two investigated soft corals showed the highest N_2 fixation rates during summer when ammonium availability in the ambient seawater was low, but water temperature and irradiance were high. This is supported by the linear regression analysis revealing for N_2 fixation a significant negative relationship to inorganic nutrients and positive correlations to water temperature and light intensity (Table 5.5). This seasonal pattern is in accordance with two recent studies on pelagic diazotrophs in the Gulf of Aqaba (Foster et al. 2009, Rahav et al. 2013). Both measured up to 6 times higher N_2 fixation rates in the photic water layer during the stratified summer and early fall months compared to the well-mixed conditions in winter and spring. High water temperature can stimulate the enzymatic activity of nitrogenase (Capone et al. 1997, 2005), while high ammonium concentrations in the water can clearly inhibit N_2 fixation as demonstrated in laboratory studies on diazotroph cultures (reviewed in Sohm et al. 2011). Roughly 25 % more energy is required to reduce N_2 (87 kcal) than NO_3^- (69 kcal)

to ammonium, therefore it is energetically inefficient to fix N_2 in marine environments with DIN concentrations above a certain threshold (~1 μM). High irradiance can inhibit the process of N_2 fixation indirectly due to enhanced photosynthetic O_2 production. However, P_{gross} of the two investigated soft corals showed only a slight increase during summer, which started already during spring when inorganic nutrient availability in the water column was highest. Thus, the strong increase in N_2 fixation during summer is most likely caused by a combination of these different environmental factors, but may be stronger influenced by the direct effects of high water temperature and low nutrient availability than by the indirect effects of light.

Corals have developed several seasonal adaptations to protect the photosystem from harmful photons during the summer months. This includes for example the down-regulation of photosynthesis by the zooxanthellae (Warner et al. 2002), the decrease of zooxanthellae abundance (Fitt et al. 2000) or the enhanced production of photoprotecting mycosporine-like amino acids (MAAs) (Michalek-Wagner 2001). The present study thus suggests that the high N_2 fixation during summer may provide the soft corals with the N compounds needed to produce MAAs during the brightest periods of the year, or to regain their zooxanthellae density during the following recovery phase.

In both soft corals R revealed a trend similar to N_2 fixation, with maximum rates during summer. This is also supported by the positive relationship between N_2 fixation and R rates, suggesting linkage between both processes. During summer, respiratory metabolism of corals generally increases as they build up biomass for reproduction (Shlesinger et al. 1998, Fitt et al. 2000). For example, in the soft coral *Heteroxenia fuscescens* (Xeniidae) from the Northern Red Sea, the biochemical tissue composition changed over the year with the highest energy content during summer, followed by spring, fall and finally winter (Ben-David-Zaslow & Benayahu 1999). This seasonal pattern reflects the high coral fecundity and reproduction in summer (Ben-David-Zaslow et al. 1999). Thus, reproduction during summer may be supported by the increased availability, and use, of N_2 fixation products. Given the low DIN availability and the low N_2 fixation activity in the water column of the Gulf of Aqaba (Foster et al. 2009, Rahav et al. 2013), as well as the importance of N for cell maintenance, growth and functioning, the association with N_2 fixing bacteria may be a key component of soft coral nutrition during summer.

Ecological implications

Corals profit from the association with several symbionts including N_2 fixing bacteria. Although the present study could not determine whether the diazotrophs are internally (i.e. as endosymbionts in the coral tissue; Lesser et al. 2004) or externally associated with the coral (i.e. in the mucus layer; Lema et al. 2012), both associations are likely to benefit the coral holobiont by providing bioavailable N. The zooxanthellae may also internally harbor diazotrophs thereby directly receiving fixed N, similarly to what has been shown for a different diazotrophs – eukaryotic algae symbiosis (Foster et al. 2011). Overall, the ability of corals to acquire N both *via* diazotrophy and *via* uptake of DIN from the surrounding seawater is advantageous in an environment where the availability of dissolved nutrients is generally low and episodic. Recently, enzymes enabling ammonium assimilation were detected in endosymbiotic algae and the coral host suggesting that both could benefit from the products of N_2 fixation (Leggat et al. 2007, Yellowlees et al. 2008, Stambler 2011). The present study suggests that uptake of DIN from the seawater may fuel coral metabolism during the nutrient-enriched spring period, while N_2 fixation products may be a key component of coral nutrition during the nutrient-depleted summer months.

Given the usual low rates of N_2 fixation in the water column of nutrient-poor coral reefs (Foster et al. 2009, Rahav et al. 2013), benthic reef organisms, including soft corals, may provide habitat for diazotrophs, thereby playing a key role for the input of new N into the reef ecosystem. In many reef locations worldwide, soft corals represent the second most dominant benthic group after scleractinian corals (Benayahu & Loya 1977, Fabricius & De'ath 2001, Inoue et al. 2013) and in the investigated study area the soft coral cover has increased by 50 % since 2007 (unpublished data), now reaching up to 21.6 % of the total benthic cover. Therefore, the present study suggests that soft corals may contribute importantly to the overall input of fixed N within the reef, although N_2 fixation rates per unit surface area are low compared to scleractinian corals. Soft corals also represent major space competitors for hard corals, and shifts from hard to soft coral dominance has been observed in several reef locations worldwide (Tilot et al. 2008, Norström et al. 2009). The data in the present manuscript thus indicate that input of new N *via* N_2 fixation may be reduced in soft coral reefs compared to those dominated by hard corals, with potential implications on biogeochemical element cycles and reef ecosystem functioning.

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Chapter VI

Inorganic nutrient availability affects organic matter fluxes and metabolic activity in the soft coral genus *Xenia*[*]

Abstract

The release of organic matter (OM) by scleractinian corals represents a key physiological process that importantly contributes to coral reef ecosystem functioning, and is affected by inorganic nutrient availability. Although OM fluxes have been studied for several dominant reef taxa, no information is available for soft corals, one of the major benthic groups in tropical reef environments. Thus, this study investigates OM fluxes along with other key physiological parameters (i.e. photosynthesis, respiration and chlorophyll *a* tissue content) in the common soft coral genus *Xenia* after a 4-week exposure period to elevated ammonium (N; 20.0 μmol l^{-1}), phosphate (P; 2.0 μmol l^{-1}) and combined inorganic nutrient enrichment treatment (N+P). Corals maintained without nutrient enrichment served as non-treated controls and revealed constant uptake rates for particulate organic carbon (POC) (-0.315 ± 0.161 mg POC m^{-2} coral surface area h^{-1}), particulate nitrogen (PN) (-0.053 ± 0.018 mg PN m^{-2} h^{-1}) and dissolved organic carbon (DOC) (-4.8 ± 2.1 mg DOC m^{-2} h^{-1}). Although DOC uptake significantly increased in the N treatment, POC flux was not affected. The P treatment significantly enhanced PN release as well as photosynthesis and respiration rates, suggesting that autotrophic carbon acquisition of zooxanthellae endosymbionts influences OM fluxes by the coral host. Our physiological findings confirm the significant effect of inorganic nutrient availability on OM fluxes and key metabolic processes for the soft coral *Xenia*, and provide the first clues on OM cycles initiated by soft corals in reef environments exposed to ambient and elevated inorganic nutrient concentrations.

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Introduction

Tropical coral reefs principally occur in oligotrophic waters with dissolved inorganic nutrient concentrations close to the limits of analytical detection. However, over the last few decades, elevated inorganic nutrient concentrations, mainly attributable to anthropogenic activities, have been reported from various coral reef environments worldwide (Bell, 1991; Dubinsky and Stambler, 1996; Lapointe and Clark, 1992; Tomascik and Sander, 1987). Elevated inorganic nutrient concentrations can seriously impact coral reef ecosystems by reducing calcification rates, reproductive success and recruitment of corals, while concomitantly promoting the growth of reef-associated benthic macroalgae (Dubinsky et al., 1990; Koop et al., 2001). This may subsequently result in a substantial loss of coral cover and ensuing benthic community phase shifts, i.e. from scleractinian coral dominance to dominance by other invertebrates (i.e. soft corals, sponges, hydrozoans and ascidians) or benthic macroalgae (Dubinsky and Stambler, 1996; Fleury et al., 2000; Hawker and Connell, 1989). Besides enhancing the growth of benthic macroalgae, elevated inorganic nutrient concentrations may also fuel coral endosymbiotic microalgae (i.e. zooxanthellae), thereby potentially influencing the symbiotic interaction (Dubinsky and Stambler, 1996). Previous studies on scleractinian coral taxa have revealed that under oligotrophic conditions zooxanthellae are nutrient-limited and contribute importantly to the coral's nutrition by translocating up to 97% of all photosynthetically fixed carbon to their host (e.g. Muscatine et al., 1984). Elevated ambient inorganic nutrient concentrations enable zooxanthellae to retain significantly more fixed carbon for their own metabolism, and consequently less of it is eventually translocated (Dubinsky and Jokiel, 1994). This reduced transfer of energy-rich photosynthates creates an insufficient diurnal energy supply in the host (Leletkin, 2000), and may consequently affect organic matter (OM) exudation, as up to 40% of carbon fixed by photosynthesis is being released into surrounding reef waters, most of it presumably as coral mucus (Crossland et al., 1980).

Exudation of mucus fulfils important functions for corals as well as the entire reef ecosystem. Mucus secretion onto the epidermal tissue surface by mucus gland cells (i.e. mucocytes) forms a protective surface mucus layer, which supports heterotrophic ciliary feeding ability, protects the coral against desiccation (Krupp, 1984), sedimentation (Riegl and Branch, 1995) and pathogens (Ritchie, 2006), as well as physical and ultraviolet-radiation-related damage (reviewed in Brown and Bythell, 2005; Drollet et al., 1993), and enhances coral resistance to changes in temperature and salinity (Marcus and Thorhaug, 1982). The protective surface mucus layer of scleractinian corals is continuously replaced, and thus large amounts of particulate (POM) and dissolved organic matter (DOM) are released into the water column (Ferrier-Pagès et al., 1998; Naumann et al., 2010; Wild et

al., 2008). There, coral-derived OM importantly sustains reef ecosystem functioning by acting as an efficient particle trap and energy carrier in biogeochemical element cycles (Naumann et al., 2009; Wild et al., 2004).

Despite our current knowledge on elevated inorganic nutrient concentrations in coral reef environments, to date only two recent studies (Naumann et al., 2010; Tanaka et al., 2010) have described their effect on OM release rates by scleractinian corals. Interestingly, both of these studies have observed reduced POM and DOM release in response to elevated inorganic nutrient concentrations. Besides effects on specific key physiological processes, such as coral-derived OM release, elevated inorganic nutrient concentrations may affect overall biogeochemical OM cycles in coral reefs as a result of bottom-up effects on benthic community structure. Beyond coral-algae phase shifts, rising inorganic nutrient concentrations possess the potential to cause alternative states in coral reefs, such as a substantial increase in soft coral abundance (reviewed in Norström et al., 2009). Although OM fluxes by scleractinian corals have recently been well described (Naumann et al., 2010; Naumann et al., 2011; Wild et al., 2004; Wild et al., 2008), our general understanding of biogeochemical OM cycles initiated by reef-associated soft coral taxa is still very limited, and no information exists regarding a potential influence by elevated inorganic nutrient concentrations.

Therefore, this laboratory study quantifies OM fluxes by the common soft coral genus *Xenia* following a 4-week exposure period to control (non-enriched) and enriched inorganic nutrient concentrations. A series of post-exposure incubation experiments conducted in non-nutrient-enriched seawater investigates variations in net fluxes of particulate organic carbon (POC), particulate nitrogen (PN) and dissolved organic carbon (DOC). These measurements are complemented by quantitative assessments of *Xenia* key physiological parameters [photosynthesis, respiration and chlorophyll *a* (chl *a*) tissue content], likewise determined after exposure to control (non-enriched) and enriched inorganic nutrient conditions.

Methods

Collection and maintenance of corals

The colony-forming soft coral *Xenia* is an abundant genus that is common in various Indo-Pacific reef ecosystems (Benayahu and Loya, 1977; Dinesen, 1983; Fishelson, 1970; Mergner and Schuhmacher, 1981; Tursch and Tursch, 1982). *Xenia* colonies were collected in 5 to 10 m water depth from a northern Red Sea reef near the city of Aqaba (Jordan) and subsequently maintained

under constant conditions in a closed aquarium system for approximately 3 years before the experiments described herein. The respective *Xenia* colonies appeared creamy coloured with monomorphic, pinnulled, pulsating and non-retractile tentacles. According to Reinicke (1997), we assume that *Xenia* specimens used in the present study belong to the species *Xenia umbellata* Lamarck 1816, which is described as a common pulsating *Xenia* species from the Red Sea. However, because of uncertainty in accurate species identification, we refer to the genus *Xenia* throughout this manuscript.

Experimental design

Experiments were carried out in the laboratory under controlled conditions. A total of 24 healthy *Xenia* colonies, each attached to a small piece of rock, were collected from the maintenance reef aquarium. For better handling, *Xenia* colonies were fixed with their attached rock onto ceramic tiles (4.6 x 4.6 cm) using conventional coral glue. A 200 l aquarium was used as an experimental tank, inside which the corals were acclimatized to the respective experimental water temperature and light intensity (i.e. 25.3 ± 0.3°C and 57.2 ± 0.6 µmol photons m^{-2} s^{-1}; mean ± s.e.m.) 2 weeks prior to experimentation. The light intensity used for experimentation was adjusted to light levels measured with data loggers (Onset HOBO Pendant UA-002-64; spectral detection range: 150–1200 nm; temperature accuracy: ± 0.53°C, Bourne, MA, USA) in the Gulf of Aqaba, Northern Red Sea (Dahab, Egypt, 13 March 2010) at ~ 5 m water depth, where many *Xenia* colonies were observed in their natural environment. A full-spectrum metal halide lamp (Oceanlight 150+, AB Aqua Medic, Bissendorf, Germany) provided constant irradiance over a daily 12 h photoperiod. Water temperature and light intensity were continuously recorded with data loggers. Light intensity (lx) readings were converted to photosynthetically active radiation (PAR; µmol quanta m^{-2} s^{-1}, wavelength 400–700 nm) using the following approximation: 1 µmol quanta m^{-2} s^{-1} = 51.2 lx (Valiela 1984).

The experiment described in the following was carried out over a period of 4 weeks. A total of 18 *Xenia* colonies (25–96 polyps per colony) were randomly assigned to one of three nutrient treatments, while six colonies served as non-nutrient-treated controls. The aquarium water used for nutrient treatments was either enriched with ammonium (N; 20.0 µmol l^{-1} NH_4^+), phosphate (P; 2.0 µmol l^{-1} PO_4^{3-}) or both inorganic nutrients (N+P; 20.0 µmol l^{-1} NH_4^+ and 2.0 µmol l^{-1} PO_4^{3-}). The respective elevated inorganic nutrient concentrations were chosen for good comparability to previous manipulative studies (Bucher and Harrison, 2000; Godinot et al., 2011).

Non-treated corals were held in local aquarium water without ammonium and/or phosphate addition. Inorganic nutrient concentrations in the aquarium water were monitored once per week using a Genesys 10 UV spectro-photometer with photometric ammonium and phosphate test kits (Spectroquant, Merck, Darmstadt, Germany). Results remained below method detection limits (concentrations: < 0.8 μmol l^{-1} NH_4^+ and < 0.1 μmol l^{-1} PO_4^{3-}) for the duration of the experiment, and thus are regarded to be within the range of *in situ* concentrations reported for the northern Gulf of Aqaba (Rasheed et al., 2002). During the experimentation period of 4 weeks, each coral colony was maintained individually in a 1 l glass beaker filled with 800 ml nutrient-enriched (treatment corals) or local aquarium water (non-treated corals) to avoid pseudo-replication within each treatment. The particular seawater solutions used for nutrient-enriched treatments were prepared separately in 12 l buckets and distributed from there, ensuring equal nutrient concentrations in all replicate glass beakers. To this end, concentrated ammonium chloride (8 mmol l^{-1} NH_4Cl) and sodium phosphate (0.8 mmol l^{-1} NaH_2PO_4) stock solutions were prepared weekly and stored refrigerated (4°C). Treatment seawater media of the desired final concentrations were subsequently prepared in the 12 l buckets from aquarium water and the respective stock solutions. The treatment or non-treatment media in all glass beakers were renewed daily in the morning. For the remainder of the day, all 24 beakers were placed in the aquarium with their rims 5 cm above the water surface. The position of each beaker was randomly changed every day to ensure comparable temperature and light conditions for each coral replicate over the entire 4-week exposure period.

Quantification of OM fluxes

To quantify OM fluxes by *Xenia* corals maintained for 4 weeks under nutrient-treated (N, P and N+P) and non-treated conditions, laboratory experiments were carried out following the established beaker incubation technique (Herndl and Velimirov, 1986). Incubation media for all individual beaker incubations of nutrient-treated and non-treated *Xenia* colonies were prepared from one large volume of non-nutrient-enriched aquarium seawater to ensure comparable incubation conditions among treatments. These concerned, in particular, inorganic nutrient and OM concentrations, as well as planktonic microbial activity of the incubation medium. After coral-attached rocks and tiles had been carefully cleaned of algal turf, each colony was placed individually into a pre-cleaned glass beaker (500 ml) after a very short air exposure (< 2 s). Beakers only filled with aquarium seawater (without coral) served as seawater controls (N = 5). After 20 min, all beakers were carefully submerged into the large volume of the experimental tank, resulting in the complete renewal of the beaker volume, which was meant to avoid elevated initial OM

concentrations possibly caused by initial coral air exposure. Thereafter, all beakers were positioned in the experimental tank with their rims 5–10 cm above the water surface to ensure equal water temperature and light conditions, as during the 4-week nutrient exposure period. All beakers were covered with transparent cellophane foil to avoid the input of airborne particles, leaving two small side openings for air exchange. The incubation media were not stirred during the relatively short-term incubation period (5–6 h) in order to rule out the potential effect of water flow on the structural composition of *Xenia*-derived organic carbon and to allow for an adequate comparison to previous studies using the same beaker incubation technique (Haas et al., 2010; Herndl and Velimirov, 1986; Naumann et al., 2010; Wild et al., 2005).

Incubations were terminated after 5–6 h by carefully removing the corals from the beakers using clean tweezers. Immediately after incubations (≤ 5 min), water subsamples were drawn from the homogenized incubation medium. For DOC, 10 ml subsamples were drawn from each beaker using clean syringes and passed through sterile polyethersulfone membrane filters (0.2 µm pore-sized, pre-washed with 6 ml sample; VWR International, Darmstadt, Germany). The filtrate was collected in pre-combusted (450°C, 6 h) glass vials, which were kept frozen at -20 °C pending analysis. Leakage of DOC from polyethersulfone filter membranes (Khan and Subramania-Pillai, 2007) was found to be insignificant, as quantified by repeated analyses of different lots of original filters following the described sampling protocol. For analysis, DOC samples were defrosted, acidified to a pH < 2 by adding 50 µl of 2 mol l^{-1} HCl, and purged with O_2 for 2 min to remove dissolved inorganic carbon. Subsequently, DOC concentrations were determined by high-temperature catalytic oxidation using a DIMA-TOC 100 total organic carbon analyzer (Dimatec Analysentechnik, Essen, Germany) with potassium hydrogen phthalate as elemental standard. After DOC sampling, O_2 concentrations in all incubation media were determined using an optode sensor (HQ 10, accuracy ± 0.05%; HACH LANGE, Düsseldorf, Germany) to confirm oxic conditions during incubation experiments. The remaining incubation medium (350–450 ml) was vacuum filtered through pre-combusted (450°C, 6 h) GF/F filters (25 mm diameter; Whatman, Maidstone, UK) in order to quantify POC and PN contents. The filters were immediately dried for at least 48 h at 40°C and kept dry until further analysis. As the presence of particulate inorganic carbon could not be ruled out by test measurements of the incubation water, samples were not treated with HCl prior to analysis. For POM analysis, dried filters were wrapped in silver foil and measured using a THERMO NA 2500 elemental analyzer (standard deviation < 3%; Thermo Fisher Scientific, Passau, Germany) with atropine and cyclohexanone-2, 4-dinitrophenylhydrazone as elemental standards.

After incubation, the number of polyps of each colony was counted and the mean surface area of a *Xenia* polyp was determined by geometric measurements on 100 completely protruded polyps randomly distributed over all 24 colonies. The diameter (2*r*, where *r* is the radius) and the length of a tentacle foot [height (*h*)] were measured using a ruler (± 0.1 cm accuracy). Thus the surface area of a *Xenia* polyp was mathematically handled as a two-sided circle ($2\ \pi r^2$) on top of a cylindrical tentacle foot ($2\ \pi rh$). The mean surface area of a polyp was subsequently multiplied by the number of polyps to generate the total surface area of each incubated *Xenia* colony. POC:PN ratios were calculated from molar contents of POC and PN of the respective coral and seawater control incubation media. As all incubations were carried out in non-nutrient-enriched aquarium seawater, mean POC, PN and DOC concentrations measured in seawater control incubations were subtracted from the concentrations measured in *Xenia*-incubated water. The results were related to the volume of the incubation medium and subsequently normalized by incubation time and colony surface area in order to calculate net OM flux rates (mg m^{-2} h^{-1}).

Physiological measurements

Following the OM incubation procedure, each coral was placed individually in a gas-tight 500 ml glass chamber completely filled with non-nutrient-enriched aquarium seawater and incubated for 2–3 h at experimental temperature and light conditions. An initial incubation was carried out in the light for net photosynthesis (P_{net}) determination, while a subsequent dark incubation without previous dark acclimatization of corals was conducted to measure dark respiration (*R*). Higher *R* rates of light- compared with dark-adapted corals (Anthony and Hoegh-Guldberg, 2003; Porter et al., 1984) provide a better approximation for gross photosynthesis (P_{gross}) calculation. Photosynthesis may show a lag response during the first minutes of dark incubation, but O_2 evolution can be regarded as negligible, if seen in the context of 2–3 h incubation time. O_2 concentrations were measured at the beginning and end of each incubation period using an O_2 optode sensor (HQ 10, accuracy ± 0.05%, HACH LANGE). To calculate P_{net} and *R* rates, O_2 end concentrations were subtracted from start concentrations and the results were normalized by incubation duration. Parallel to the above incubations, five seawater controls (without corals) were incubated to measure planktonic background metabolism. As all incubations were carried out in non-nutrient-enriched seawater, P_{net} and *R* rates obtained from seawater control incubations could finally be subtracted from the rates obtained from coral incubations. These rates were related to incubation volume and normalized to coral surface area. P_{gross} rates were calculated according to $P_{gross} = P_{net} + R$. As light *R* rates during active photosynthesis may be significantly higher than dark

R rates (Fabricius and Klumpp, 1995), the presented P_{gross} rates are conservative estimates based on dark respiration rates.

After incubations, three polyps from each colony were cut off using clean scissors. The tissue material was washed four times with pure water to remove all salt before the samples were freeze-dried (Lyovac GT2/ GT2-E, SRK Systemtechnik, Riedstadt-Goddelau, Germany) for dry mass measurement. For pigment extraction, 10 ml of 90 % acetone was added and samples were stored in the dark for 24 h pending analysis. Total pigment contents of chl *a* and phaeophytin were measured by fluorimetry (TD-700, GAT-Gamma Analysentechnik, Bremerhaven, Germany) according to Holm-Hansen et al. (Holm-Hansen et al., 1965). After an initial measurement, 24 µl of HCl were added to the extract to convert chl *a* to phaeophytin followed by a measurement 2 min after. This procedure yielded the phaeophytin-free chl *a* content, which was finally normalized by *Xenia* tissue dry mass.

Data analysis

All statistical analyses were carried out using the Statistica software package (StatSoft, Hamburg, Germany). Prior to analyses, outlier values within the individual treatments were identified using Dean-Dixon tests and data were tested for homogeneity of variances and normality using Levene and Shapiro-Wilk tests. If preconditions for parametric analysis were fulfilled, one-way ANOVAs were performed followed by Tukey's *post hoc* tests for between-group comparisons. If data did not meet parametric assumptions, non-parametric Kruskal-Wallis tests were used. The individual differences among the four groups were evaluated using Kruskal-Wallis multiple-comparison Z-tests with Bonferroni adjustment for multiple comparisons. All statistical tests were evaluated at an alpha level of 0.05.

Results

OM fluxes

Table 6.1 provides an overview on DOC, POC and PN concentrations as well as on POC:PN ratios in the incubation media measured at the end of all coral and seawater control incubations in non-nutrient-enriched aquarium seawater (means ± s.e.m.). Normalized flux rates (Fig. 6.1, means ± s.e.m.) calculated after subtraction of seawater control values revealed an uptake of DOC (-4.8 ± 2.1 mg DOC m^{-2} coral surface area h^{-1}) and POM (-0.315 ± 0.161 mg POC m^{-2} h^{-1}, -0.053 ± 0.018 mg PN m^{-2} h^{-1}) by non-treated corals maintained for 4 weeks under non-nutrient-enriched conditions.

Exposure to elevated inorganic nutrient concentrations over 4 weeks significantly affected DOC fluxes by *Xenia* (Kruskal-Wallis test, $H = 8.28$, d.f. = 3, $P = 0.041$; Fig. 6.1A). DOC fluxes of nutrient-treated corals were not significantly different from those of non-treated corals (Kruskal-Wallis multiple-comparison, $P > 0.05$), whereas differences between the nutrient treatments were evident. Analysis revealed significantly higher DOC uptake rates of N-treated (-7.9 ± 0.5 mg DOC m^{-2} surface area h^{-1}; Kruskal-Wallis multiple-comparison, $P = 0.042$) compared with P-treated corals (-2.6 ± 0.9 mg DOC m^{-2} h^{-1}). DOC uptake of N+P-treated corals (-7.4 ± 1.2 mg DOC m^{-2} coral surface area h^{-1}) also increased compared to P-treated corals, albeit this was not statistically significant (Kruskal-Wallis multiple-comparison, $P > 0.05$).

Inorganic nutrient exposure also affected POM fluxes as well as POC:PN ratios in the incubation water. After subtraction of seawater controls, significant differences in POM fluxes became evident for PN (Kruskal-Wallis test, $H = 10.01$, d.f. = 3, $P = 0.019$), whereas no differences were found for flux rates of POC (Kruskal-Wallis test, $H = 3.12$, d.f. = 3, $P = 0.37$; Fig. 6.1B, C). This revealed significantly enhanced PN release rates (Kruskal-Wallis multiple-comparison, $P = 0.015$) by P-treated corals (0.052 ± 0.023 mg PN m^{-2} coral surface area h^{-1}) compared with non-treated corals (-0.053 ± 0.018 mg PN m^{-2} h^{-1}). In contrast, N (-0.023 ± 0.015 mg PN m^{-2} h^{-1}) and N+P (0.002 ± 0.004 mg PN m^{-2} h^{-1}) treatments exhibited no effect on PN fluxes (Kruskal-Wallis multiple-comparison, $P > 0.05$). The POC:PN ratio in the incubation water showed significant differences between the treatments (one-way ANOVA, $F = 13.95$, d.f. = 3, $P < 0.001$; Table 6.1), with significantly reduced ratios in the incubation water of P-treated corals (7.3 ± 0.4) compared with N-treated (9.2 ± 0.3; *post hoc* Tukey's test, $P = 0.003$), N+P-treated (8.8 ± 0.4; *post hoc* Tukey's test, $P = 0.016$) and non-treated corals (10.0 ± 0.2; *post hoc* Tukey's test, $P < 0.001$).

Physiological measurements

Chl *a* tissue content of non-treated *Xenia* colonies averaged 0.11 ± 0.01 µg chl *a* mg^{-1} tissue dry mass and was significantly affected after 4 weeks exposure to elevated inorganic nutrient concentrations (one-way ANOVA, $F = 19.77$, d.f. = 3, $P < 0.001$). Compared with non-treated corals, P-treated (0.22 ± 0.02 µg Chl *a* mg^{-1}) and N+P-treated corals (0.25 ± 0.02 µg Chl *a* mg^{-1}) showed significantly higher chl *a* tissue content (*post hoc* Tukey's test, $P < 0.001$), whereas the N treatment (0.14 ± 0.01 µg Chl *a* mg^{-1}) revealed no effect (Fig. 6.2). Comparison between the treatments showed significantly higher chl *a* content of P-treated (*post hoc* Tukey's test, $P = 0.003$) and N+P-treated corals (*post hoc* Tukey's test, $P < 0.001$) compared with N-treated corals.

Rates of P_{gross} averaged 21.6 ± 2.2 mg O_2 m^{-2} coral surface area h^{-1}, and *R* rates were 9.3 ± 0.5 mg O_2 m^{-2} h^{-1} for non-treated *Xenia* (Fig. 6.3). Exposure to elevated inorganic nutrient concentrations significantly affected *Xenia* P_{gross} (one-way ANOVA, $F = 8.84$, d.f. = 3, $P < 0.001$) and *R* rates (one-way ANOVA, $F = 5.44$, d.f. = 3, $P = 0.007$). Compared with non-treated corals, P-treated *Xenia* showed significantly higher P_{gross} (40.8 ± 3.6 mg O_2 m^{-2} h^{-1}; *post hoc* Tukey's test, $P = 0.005$) and *R* rates (14.3 ± 0.8 mg O_2 m^{-2} h^{-1}; *post hoc* Tukey's test, $P = 0.033$), whereas no significant change was observed for N-treated (P_{gross}: 17.6 ± 2.9 mg O_2 m^{-2} h^{-1}, *R*: 8.1 ± 0.8 mg O_2 m^{-2} h^{-1}) and N+P-treated corals (P_{gross}: 31.0 ± 4.6 mg O_2 m^{-2} h^{-1}, *R*: 12.0 ± 2.0 mg O_2 m^{-2} h^{-1}; *post hoc* Tukey's test, $P > 0.05$). Among nutrient treatments, P_{gross} (*post hoc* Tukey's test, $P < 0.001$) and *R* rates (*post hoc* Tukey's test, $P = 0.008$) were significantly higher for P-treated than N-treated corals.

The P_{gross}/R ratio (Fig. 6.3) of non-treated corals (2.3 ± 0.2) was nearly identical to that of N-treated corals (2.2 ± 0.2). Although the P_{gross}/R ratio of P-treated (2.8 ± 0.1) and N+P-treated corals (2.6 ± 0.2) increased compared with non-treated corals, these differences were not statistically significant (Kruskal-Wallis test, $H = 3.78$, d.f. = 3, $P = 0.29$).

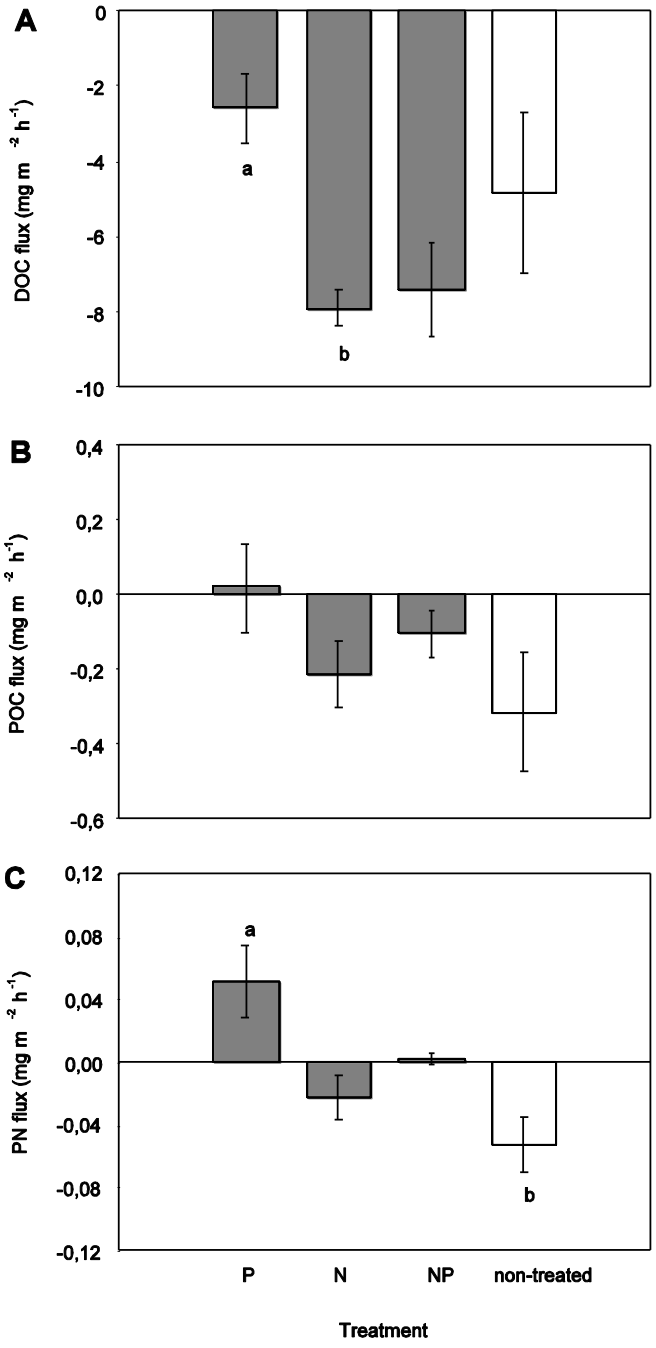

Figure 6.1. (A) Dissolved (DOC) and (B) particulate organic carbon (POC) and (C) particulate nitrogen (PN) fluxes of nutrient-treated and non-treated *Xenia* corals. Values are given as means ± s.e.m. of N = 5–6 replicates after seawater control correction (positive values indicate net release and negative values indicate net uptake). P, phosphate addition; N, ammonium addition; N+P, combined ammonium and phosphate addition; non-treated, no inorganic nutrient addition. Columns marked with 'a' are significantly different from columns marked with 'b' (one-way ANOVA, post hoc Tukey's test and Kruskal-Wallis multiple-comparison Z-test with Bonferroni adjustment, $P < 0.05$).

Table 6.1. Dissolved (DOC) and particulate (POC) organic carbon and particulate nitrogen (PN) concentrations as well as POC:PN ratios in *Xenia* incubation water and seawater controls measured at the end of beaker incubation experiments. Values are given as means ± s.e.m. of *N* = 5–6 replicates. P, phosphate addition; N, ammonium addition; N+P, combined ammonium and phosphate addition; non-treated, no inorganic nutrient addition.

Treatment	DOC	POC	PN	POC:PN
	(µM)			
P	277 ± 15	20 ± 2	2.82 ± 0.29	7.3 ± 0.4
N	241 ± 6	18 ± 1	1.98 ± 0.13	9.2 ± 0.3
NP	234 ± 7	19 ± 1	2.17 ± 0.04	8.8 ± 0.4
non-treated	272 ± 23	17 ± 2	1.69 ± 0.18	10.0 ± 0.2
Seawater control	319 ± 36	20 ± 2	2.16 ± 0.21	9.3 ± 0.2

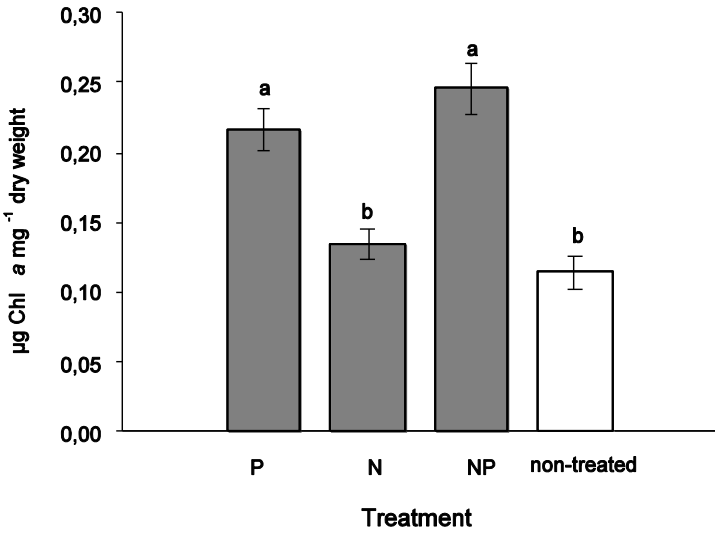

Figure 6.2. Chlorophyll *a* (chl *a*) tissue content of nutrient-treated and non-treated Xenia corals. Values are given as means ± s.e.m. of N = 6 replicates after seawater control correction. P, phosphate addition; N, ammonium addition; N+P, combined ammonium and phosphate addition; non-treated, no inorganic nutrient addition. Columns marked with 'a' are significantly different from columns marked with 'b' (one-way ANOVA, post hoc Tukey's test, $P < 0.05$)

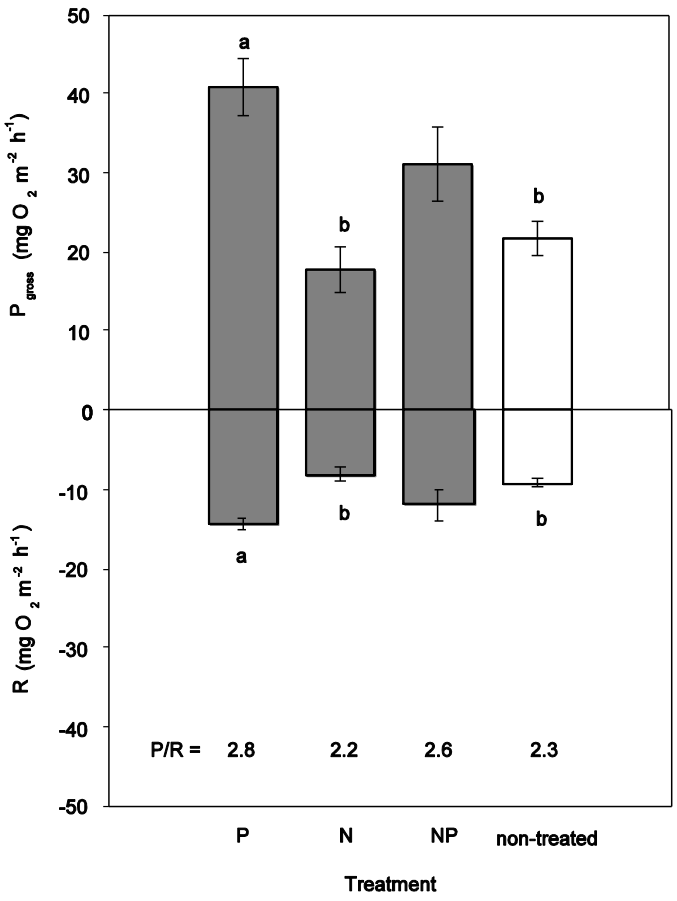

Figure 6.3. Gross photosynthesis (P_{gross}) and respiration (R) rates of nutrient-treated and non-treated *Xenia* corals. Values are given as means ± s.e.m. of N = 6 replicates after seawater control correction. P, phosphate addition; N, ammonium addition; N+P, combined ammonium and phosphate addition; non-treated, no inorganic nutrient addition. Columns marked with 'a' are significantly different from columns marked with 'b' (one-way ANOVA, post hoc Tukey's test, $P < 0.05$). Pgross/R ratios are given for each of the specific treatments.

Discussion

Our results of laboratory-based incubation experiments involving specimens of the common soft coral *Xenia* provide the first information on OM flux rates by reef-associated soft corals, one of the key benthic groups in tropical coral reef environments. In addition to presenting flux rates of soft-coral-derived OM species (POC, PN and DOC) determined under non-nutrient-enriched control conditions, we show evidence for the significant effect of mid-term exposure (4 weeks) to enriched inorganic nutrient (ammonium and phosphate) concentrations complemented by integrated measurements of key physiological parameters (P_{gross}, *R* and chl *a* tissue content). These physiological findings confirm the significant effect of inorganic nutrient availability on OM fluxes and key metabolic processes for the soft coral *Xenia*, and provide the first insights into the contribution of soft corals to OM budgets within reef environments exposed to ambient and elevated inorganic nutrient concentrations.

OM fluxes under non-enriched inorganic nutrient conditions

Recent studies have advanced our understanding of OM release and uptake processes performed by scleractinian corals in tropical reef environments (Houlbrèque and Ferrier-Pagès, 2009; Naumann et al., 2010; Wild et al., 2004). However, these processes are still unexplored for reef-dwelling soft corals, including the common taxon *Xenia*. Corals of this genus possess a high abundance of mucocytes and harbor POM in their gastrovascular cavities, which indicates their physiological ability to both release and uptake POM from the surrounding seawater (Al-Sofyani and Niaz, 2007; Fabricius and Dommisse, 2000; Lewis, 1982). Our findings revealed net POM uptake for *Xenia* colonies exposed to non-nutrient-enriched conditions. This stands in contrast to previously investigated benthic reef taxa (scleractinian corals, fire corals, reef algae, and jellyfish) from the northern Red Sea showing net POM release under ambient inorganic nutrient concentrations (Table 6.2). Coral-derived POM release fulfils important ecological functions *via* efficient particle trapping and by the concomitant recycling of energy and essential nutrients within the reef ecosystem (Wild et al., 2004; Wild et al., 2011). We therefore conclude that the soft coral *Xenia* may not contribute to ecosystem engineering *via* POM release as found for the majority of scleractinian corals.

Similar to POM fluxes, *Xenia* showed net DOC uptake under non-nutrient-enriched conditions. Uptake of DOC represents a common feeding mode in cnidarians and is known from numerous scleractinian taxa (Ferrier, 1991; Ferrier-Pagès et al., 1998; Naumann et al., 2010).

Heterotrophic DOM uptake has been shown to contribute significantly to the daily metabolic demand of the zooxanthellate soft coral *Heteroxenia fuscescens* (Schlichter, 1982), and likely also represents an important carbon source for *Xenia*. In the present study, DOC uptake rates by *Xenia* (-4.8 ± 2.1 mg DOC m^{-2} coral surface area h^{-1}) were in the range described for the scleractinian coral genera *Fungia* (-14.2 ± 5.5 mg DOC m^{-2} h^{-1}) and *Stylophora* (-14.1 ± 12.8 mg DOC m^{-2} h^{-1}), and for the jellyfish *Cassiopea* sp. (-1.2 ± 4.4 mg DOC m^{-2} h^{-1}) occurring in the northern Red Sea (Naumann et al., 2010; Niggl et al., 2010). Nevertheless, the majority of scleractinian corals as well as other benthic reef taxa investigated by previous studies net released DOC (Haas et al., 2010; Naumann et al., 2010; Niggl et al., 2010) (Table 6.2).

The observed differences in net OM fluxes between *Xenia* and several previously investigated scleractinian coral species may result from specific differences regarding each respective coral's nutrition. Soft corals are described to rely more on heterotrophic feeding due to their lower photosynthetic productivity compared with scleractinian corals. This has been demonstrated by lower ratios of P_{gross}/R ratios in soft corals (1.0–1.3) compared with scleractinian corals (2–4) (Fabricius and Klumpp, 1995; Mergner and Svoboda, 1977). In the present study, the P_{gross}/R ratio of non-treated *Xenia* corals (2.3 ± 0.2) was higher than that previously described for soft corals (e.g. *Xenia* spp., *Efflatounaria* sp. and *Sarcophyton* spp.), but still in the lower range compared with scleractinian corals (e.g. *Pocillopora damicornis* and *Fungia scutaria*). Therefore, our findings indicate that *Xenia* may indeed be more dependent on heterotrophic carbon acquisition *via* OM uptake from the surrounding seawater compared with scleractinian coral taxa.

Enrichment effects on DOC fluxes

This study is the first to investigate the effect of single and combined elevated inorganic nutrient concentrations on DOC fluxes by corals under controlled laboratory conditions. Only one previous study has looked at the effect of a combined addition of nitrate and phosphate (10 µmol l^{-1} NO_3^- and 0.5 µmol l^{-1} PO_4^{3-}) on DOM fluxes by the scleractinian coral *Montipora digitata* (Tanaka et al., 2010). Our results indicate increased DOC uptake in N-treated compared with P-treated *Xenia* corals after the 4-week exposure period. This increase may be attributed to the coral's metabolic activity, as P_{gross} rates in N-treated corals were significantly lower than those of P-treated corals. Lower P_{gross} rates in the N treatment may indicate that less carbon was available for translocation from zooxanthellae to the soft coral host (Ferrier-Pagès et al., 2000). Compared with the P treatment, N+P-treated corals showed no changes in P_{gross} rates or DOC fluxes, while non-treated corals exhibited reduced P_{gross} rates, without differences in DOC fluxes. Thus, we conclude

that besides reduced P_{gross} rates in the N treatment, elevated ammonium concentrations were mainly responsible for stimulating DOC uptake. Under low ambient inorganic nutrient concentrations, zooxanthellae metabolism and growth are limited with respect to nitrogen, and carbon is photosynthetically fixed in excess. This surplus in carbon is translocated to the host together with small amounts of nitrogen (Muscatine et al., 1984; Stimson and Kinzie, 1991; Tanaka et al., 2006). At elevated inorganic nitrogen levels in ambient seawater, zooxanthellae are enabled to retain more carbon for their own metabolism and growth, and consequently less of it is being translocated to their host (Dubinsky and Jokiel, 1994). The coral may balance this carbon deficiency by increased heterotrophic DOC uptake from the surrounding seawater, as likely observed here. Tanaka et al. (Tanaka et al., 2010) found no change in DOC release rates normalized by surface area of *M. digitata* after exposure to elevated nitrate and phosphate concentrations, but DOC release decreased if related to chl *a* tissue content. Likewise, DON flux direction in the study of Tanaka et al. (Tanaka et al., 2010) changed from release (0.40 ± 0.16 nmol DON cm^{-2} coral surface area h^{-1}) to uptake (-0.32 ± 0.17 nmol DON cm^{-2} h^{-1}). Like the mentioned change in DON flux direction, our findings of increased DOC uptake may result from the same physiological mechanism involving reduced carbon and nitrogen translocation from zooxanthellae to the host under excess inorganic nutrient supply. Ferrier-Pagès et al. (Ferrier-Pagès et al., 1998) demonstrated higher DOC release rates for fed than for starved specimens of *Galaxea fascicularis* and described DOM fluxes of corals as an indicator of their trophic status. In the present study, ammonium addition may have caused a nutritional status comparable to starvation due to reduced carbon supply *via* the zooxanthellae. Therefore, our findings suggest that *Xenia* most likely balances a resulting carbon deficiency by increasing DOC uptake.

Enrichment effects on POM fluxes

POM fluxes by *Xenia* corals were not affected in N and N+P treatments, but were mainly influenced by exposure to elevated phosphate concentrations. Under these conditions, PN release significantly increased compared with non-treated corals, and consequently caused decreased POC:PN ratios in the incubation water. Previous studies have shown that corals respond to change in environmental parameters, such as light intensity and temperature, by increasing rates of POM release to the surrounding seawater and/or by expelling their zooxanthellae endosymbionts (Brown, 1997; Brown and Bythell, 2005; Crossland et al., 1980). However, POM release by corals in response to variability in inorganic nutrient concentrations has only been described by one previous study. Naumann et al. (Naumann et al., 2010) found decreased POC and PN release rates in several

scleractinian coral genera in response to seasonally increased nitrate concentrations (seasonal range: 0.14–0.83 μmol l^{-1} NO_3^-), whereas seasonal variability in concentrations of ammonium (0.21–0.31 μmol l^{-1} NH_4^+) and phosphate (0.03–0.07 μmol l^{-1} PO_4^{3-}) showed no effect. This is in contrast to our findings, but the results are hardly comparable, as *in situ* concentrations of ammonium and phosphate are obviously significantly lower in Red Sea reef waters than concentrations used for enrichment here (20.0 μmol l^{-1} NH_4^+ and 2.0 μmol l^{-1} PO_4^{3-}).

We suggest that increased PN release within the P treatment may be strongly related to the observed physiological changes in the coral–zooxanthellae symbiosis, as P-treated corals revealed significantly higher P_{gross} rates and higher P_{gross}/R ratios than non-treated corals. Our calculated P_{gross} rates represent conservative estimates among all treatments, thus allowing an accurate treatment comparison of the physiological response to variable inorganic nutrient concentrations. Changes in P_{gross} rates and P_{gross}/R ratios can serve as indicators of the amount of photosynthates potentially available for translocation from zooxanthellae to the host (Ferrier-Pagès et al., 2000). Endosymbiotic zooxanthellae are the primary site for inorganic nutrient (e.g. ammonium and phosphate) assimilation from the surrounding seawater (Godinot et al., 2011; Grover et al., 2002) and play an important role in processes controlling the amount and composition of coral-derived OM release (Brown and Bythell, 2005). As up to 40% of net photosynthetically fixed carbon is released as mucus POC or DOC, increased photosynthesis in corals may consequently enhance POM release, as observed in the present study. Tanaka et al. (Tanaka et al., 2006) demonstrated that the carbon to nitrogen ratio of translocated photosynthetic products from zooxanthellae to the host decreases in response to elevated ambient inorganic nutrient concentrations. This may simultaneously affect the composition of OM released by the coral and is in line with our findings, which show significantly enhanced PN release and reduced POC:PN ratios in incubation media of P-treated corals. In contrast, P_{gross} rates in N- and N+P-treated corals remained unaffected, likely explaining why POM release by these specimens showed no detectable increase compared with non-treated corals.

Our data strongly indicate that the corals during experimentation were more limited by the availability of inorganic phosphate than by ammonium. Previously, it has been demonstrated that elevated ambient ammonium as well as phosphate concentrations may trigger a significant increase in photosynthesis, chl *a* tissue content and zooxanthellae abundance in several scleractinian species (Bucher and Harrison, 2000; reviewed by Davy et al., 2012; Dubinsky et al., 1990; Ferrier-Pagès et al., 2000; Godinot et al., 2011). Elevated P_{gross} rates by P-treated *Xenia* corals are most likely caused by increased chl *a* tissue content. If we assume a higher algae density in P-treated corals as a result

of increased photosynthesis and chl *a* tissue content, *Xenia* may react to an overpopulation of zooxanthellae by expelling these cells into the surrounding water. Expulsion of algae from cnidarian hosts has been described as one primary regulator process for symbiont population density, as observed, among other anthozoans, in the soft coral *Xenia macrospiculata* (Baghdasarian and Muscatine, 2000; Hoegh-Guldberg et al., 1987; Stimson and Kinzie, 1991). As zooxanthellae generally posses a lower carbon to nitrogen ratio (5.9) than free-living phytoplankton organisms (6.6) (Belda et al., 1993), zooxanthellae expulsion may provide another explanation for the observed increase in PN release and the reduced POC:PN ratio found in the incubation water of P-treated corals.

Ecological implications

Our findings demonstrate that the soft coral *Xenia* shows no OM net release under oligotrophic conditions, while supplementary enrichment of inorganic nutrient concentrations only results in comparably minor to insignificant POM release rates. This is in contrast to the majority of previously investigated scleractinian coral, algae and jellyfish taxa releasing comparably high amounts of POM and/or DOM. In oligotrophic coral reef environments, usually dominated by scleractinian corals, the role of *Xenia* in biogeochemical OM cycles may thus generally represent a net sink for OM.

These findings are of special interest, as soft corals represent dominant taxa within the benthic community of many reefs, as well as major space competitors for scleractinian corals because of their ability to quickly colonize vacant reef substrate. Moreover, soft coral dominance over OM-releasing scleractinian coral taxa, as a consequence of local disturbances, has been documented from various global reef locations (Fox et al., 2003; reviewed in Norström et al., 2009; Tilot et al., 2008). Norström et al. (Norström et al., 2009) suggested that such shifts in benthic reef community composition, which coincide with a substantial decrease of scleractinian coral cover and loss of their important role as reef ecosystem engineers, are frequently associated with changes in the dynamics of bottom-up factors (e.g. inorganic nutrient enrichment). Taking this into account, our current findings strongly indicate that the important contribution of coral-derived OM release to biogeochemical element cycles may be greatly reduced under eutrophic conditions in reef ecosystems dominated by *Xenia* or other soft coral taxa, consequently causing a decline in coral ecosystem engineering with concomitant implications for overall ecosystem functioning (Wild et al., 2011).

Besides OM release, the three-dimensional reef framework built by scleractinian corals provides habitat for a highly associated biodiversity (Wild et al., 2011), while its biological and physical erosion generates calcareous reef sands acting as important biocatalytical filter systems, enhancing nutrient- and OM-recycling processes supported by an abundant heterotrophic microbial community (Wild et al., 2004; Wild et al., 2006). As soft corals only marginally contribute to calcareous reef accretion (Jeng et al., 2011), they are not a substitute for the scleractinian ecosystem engineering capacities, which consequently may not be sustained in soft-coral-dominated reef communities, likely resulting in ecosystem degradation. Nevertheless, *in situ* studies in scleractinian- and soft-coral-dominated reef ecosystems are required to increase our understanding of these dynamic processes and provide important insights into the potential changes of reef ecosystem functioning after phase shifts from scleractinian to soft coral dominance.

Table 6.2. Dissolved (DOC) and particulate (POC) organic carbon and particulate nitrogen (PN) fluxes for various classes of benthic reef organisms in comparison with non-nutrient-treated *Xenia* soft corals. Rates are given as means ± s.e.m. for five scleractinian coral genera (*Acropora, Fungia, Goniastrea, Pocillopora and Stylophora*), nine benthic reef algae genera (i.e. *Caulerpa, Peyssonnelia* and turf algae), one hydrozoan genus (*Millepora*) and one scyphozoan genus (*Cassiopea* sp.). Positive values indicate net release and negative values indicate net uptake.

Study site	Organism	DOC	POC	PN	Method	Reference
		(mg m^{-2} h^{-1})				
Red Sea	Scleractinian corals	-20.7 ± 21.2	2.8 ± 0.3	0.29 ± 0.03	Beaker	Naumann et al. 2010
Red Sea	Reef algae	14.5 ± 2.3	5.1 ± 0.5	0.35 ± 0.03	Beaker	Haas et al. 2010
Red Sea	Fire coral	9.2 ± 12.8	0.3 ± 0.1	0.04 ± 0.01	Beaker	Naumann et al. 2010
Red Sea	Jellyfish	-1.2 ± 4.4	21.0 ± 9.0	2.3 ± 1.1	Beaker	Niggl et al. 2010
Laboratory	Soft coral	-4.8 ± 2.1	-0.3 ± 0.2	-0.05 ± 0.02	Beaker	Present study

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Chapter VII

The effect of light availability on dinitrogen fixation associated with scleractinian corals along a depth-gradient in the Northern Red Sea*

Abstract

Light availability in coral reefs has a major influence on photosynthesis (PS) and dinitrogen fixation (NF) activity associated with scleractinian corals. Several photo-adaptive mechanisms are known to sustain high primary productivity under variable light regimes, but the light-dependency of NF and its potential physiological relationship to PS has received considerably less attention. Therefore, the present study simultaneously investigated PS and NF of two dominant scleractinian corals (*Acropora* sp. and *Stylophora* sp.) along a depth-mediated light gradient from 5 m (680 ± 140 µmol m^{-2} s^{-1}) down to 20 m (110 ± 10 µmol m^{-2} s^{-1}) water depth in a northern Red Sea fringing reef. In addition, coral samples were analyzed for $\delta^{15}N$ signatures, zooxanthellae density and chlorophyll *a* content. Findings revealed that PS and NF remained constant along the depth gradient, although NF associated with *Stylophora* sp. slightly decreased with increasing water depth. However, corals from deeper waters possessed a depleted $\delta^{15}N$ signature thus indicating a higher usage of NF products that may in turn support the corals' observed photo-adaptation (increased zooxanthellae density and chlorophyll *a* content) to low light availability. Overall, this study suggests that the physiological stability in corals along the depth gradient may be found in the mutual interplay and trade-off of PS and NF products between zooxanthellae and diazotrophs leading to an optimal adaptation of the involved symbiotic partners to different environmental light conditions.

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Introduction

The light availability in coral reefs has a major influence on the physiology, ecology and distribution of zooxanthellate corals. Light intensity decreases exponentially with water depth, but corals sustain their photoautotrophic performance through several photoacclimative processes. These include changes in algal density, algal pigmentation, coral morphology or photo-behavior leading to optimized light protection, light harvesting and photosynthetic efficiency under different light availabilities (Dubinsky et al. 1984, Levy et al. 2003, Mass et al. 2007).

In shallow waters scleractinian corals are nearly transparent and derive up to 95 % of their metabolic needs from carbon (C) translocated by their photosynthesizing zooxanthellae (Dubinsky and Jokiel 1994). The high photosynthetic C acquisition and primary production by shallow water corals requires sufficient fixed nitrogen (N) sources which is in an oligotrophic environment, such as coral reefs, not provided by N availability in the surrounding waters (Dubinsky and Falkowski 2011). Thus, these corals depend on an additional N supply, potentially *via* dinitrogen (N_2) fixing symbionts (diazotrophs). The mutualistic relationship with N_2 fixing bacteria has evolved in several reef organisms as an adaptation to the oligotrophic environmental conditions (Lema et al. 2012, Lesser et al. 2004). In corals, zooxanthellae primarily use the N fixed by the diazotrophs, while the latter receives the energy for N_2 fixation from translocated C fixed by the zooxanthellae (Shashar et al. 1994, Lesser et al. 2007). While the adaptation mechanisms of corals and their C fixing zooxanthellae along depth gradients have been intensively studied, changes in their N_2 fixation activity has received less attention.

Light availability indirectly regulates N_2 fixation through enhanced photosynthesis. More photosynthetically fixed C potentially stimulates N_2 fixation by providing the required energy, while the enhanced photosynthetic oxygen (O_2) production inhibits the synthesis of nitrogenase, the enzyme responsible for N_2 fixation (Stal 2009). Thus some phototrophic diazotrophs (cyanobacteria) have evolved specialized cells (heterocysts) that spatially separate the O_2-sensitive process of N_2 fixation from the O_2-evolving photosystem II (Stal 2009). Conversely, non-heterocystous cyanobacteria avoid O_2 inhibition by fixing N_2 preferentially at night. N_2 fixation associated with scleractinian corals has been studied on a diel cycle and highest rates occur during the twilight period (Lesser et al. 2007). Moreover, the abundance of corals associated with diazotrophs increases with increasing water depth (Lesser et al. 2007), and corals from greater water depth possess a reduced $\delta^{15}N$ isotopic signature compared to corals from shallow areas (Alamaru et al. 2009, Muscatine and Kaplan 1994). The natural abundance of stable N isotopes ($\delta^{15}N$) in reef organisms can be used as an indicator for N_2 fixation activity (Kayanne et al. 2005) as it reflects the

nature of the N source which is taken up and accumulated by an organism. While enriched $\delta^{15}N$ signatures (8 to 22 ‰) indicate the influence of upwelling or sewage in coastal waters (Heikoop et al. 2000, Yamamuro et al. 2003), a depleted $\delta^{15}N$ signature (-2 to 2 ‰) rather results from exposure to inorganic fertilizers or atmospheric N_2 fixation as atmospheric N_2 has a $\delta^{15}N$ value of 0 ‰ (Yamamuro et al. 1995, France et al. 1998, Kayanne et al. 2005). Kayanne et al. (2005) reported a significant negative correlation between the $\delta^{15}N$ signal and N_2 fixation in microbial mats. Parallel measurements of $\delta^{15}N$ signatures and N_2 fixation activity are helpful to evaluate the nutrient status and the net contribution of fixed N to the organism, but so far this approach has rarely been applied (Kayanne et al. 2005, Lesser et al. 2007). Furthermore, to our knowledge no study has simultaneously investigated the light-dependency of coral-associated primary productivity and N_2 fixation. Thus, the aim of the present study was to investigate N_2 fixation activity (via acetylene reduction) and primary productivity (via O_2 fluxes) along with other potential light-mediated response parameters ($\delta^{15}N$ signature of coral tissue and zooxanthellae, zooxanthellae density and chlorophyll (chl) *a* content) of two dominant scleractinian corals (*Acropora* sp. and *Stylophora* sp.) collected from 5 to 20 m water depth in the Northern Gulf of Aqaba (Red Sea). In order to understand if N_2 fixation products may support the photo-adaptation of corals to different light regimes, a potential relationship between coral-associated N_2 fixation and photosynthesis along the depth-mediated light gradient was evaluated.

Methods

Study site and coral collection

This study was carried out during October 2013 at a fringing coral reef located within a marine reserve in front of the Marine Science Station (MSS) at the Northern Gulf of Aqaba, Jordan (29° 27`N, 34° 58`E). Two hard coral genera (*Acropora* sp. and *Stylophora* sp.) were collected by carefully chiseling fragments from the reef at 5, 10 and 20 m (n = 7 - 8) using SCUBA. All samples were transported back to the MSS within 60 min of collection and individually fixed onto ceramic tiles using a two-part epoxy putty (Reef Construct, AQUA MEDIC GmbH, Bissendorf, Germany). Coral fragments were placed in an outdoor 800 L experimental tank supplied with seawater pumped directly from the reef (exchange rate: 4000 L h^{-1}) to ensure additionally *in situ* water temperature and nutrient concentrations. *In situ* light intensity was measured as photosynthetically active radiation at 5, 10 and 20 m using an underwater quantum sensor (LI-COR LI-192SA), and replicated in the experimental tank using black netting (5 m: 680 ± 140, 10 m: 400 ± 40, 20 m: 110

± 10 µmol m^{-2} s^{-1}, mean ± SD). After 1 week of recovery from fragmentation the following incubations were conducted in the experimental tank under maintenance conditions.

Oxygen flux measurements

Two individual O_2 flux incubations were conducted to quantify respiration (R) rates of the corals 2 h after sunset in complete darkness and maximum net photosynthesis (P_{net}) the following day around 12:00 during the most stable and maximum light intensities. Each fragment was incubated for 90 min in a respirometric gas-tight glass chambers (1 L) under constant stirring (600 rpm; Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific™). Additional chambers (1 L, n = 7) filled only with seawater served as controls to measure planktonic background metabolism. O_2 concentrations were measured at the beginning and end of each incubation period using a salinity- and temperature-corrected O_2 optode sensor (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). To calculate O_2 fluxes, O_2 start concentrations were subtracted from end concentrations, and the results were corrected for the seawater control signal, related to incubation volume and normalized to incubation time and coral surface area. Finally, gross photosynthesis (P_{gross}) was calculated according to $P_{gross} = P_{net} - R$.

Acetylene reduction assays

Acetylene reduction as a proxy for N_2 fixation was quantified for each sample 3 - 4 h after the end of the P_{net} incubation period by applying the modified acetylene (C_2H_2) reduction technique (Capone 1993, Wilson et al. 2012). C_2H_2 gas was freshly generated from calcium carbide and bubbled through fresh seawater in order to produce C_2H_2-enriched seawater. Each incubation chamber contained 80 % seawater and 20 % air in headspace, and was equipped with a spring-loading glass lid with a rubber injection port on top for gas sampling. Experiments were started by replacing 10 % of the seawater with C_2H_2-enriched seawater and 10 % of the air with C_2H_2 gas after sealing the incubation chambers gastight. Additional seawater control chambers (without corals) were tested for planktonic background C_2H_2 reduction to ethylene (C_2H_4). All chambers were incubated over 24 h under constant stirring (600 rpm). One ml gas samples were withdrawn after 0, 4, 12, 16 and 24 h with gastight syringes, transferred into gastight 2 ml vials previously filled with distilled water, and stored frozen upside down until analysis. C_2H_4 concentrations of gas samples were measured in the field laboratory using a reducing compound photometer (RCP) (Peak

Laboratories) with a detection limit of 100 ppb. Calibration of the RCP was conducted using serial dilutions of a 200 ± 4 ppm C_2H_4 standard in air (Restek, Bellefonte, PA, USA). The C_2H_4 evolution in each incubation chamber was calculated according to Breitbarth et al. (2004). Values were finally corrected for the unfiltered seawater control signal and normalized to incubation time and coral surface area. All rates are reported as C_2H_4 production rates (nmol C_2H_4 cm^{-2} h^{-1}) to allow for good comparison to previous studies using the C_2H_2 reduction assay.

Chl a content and zooxanthellae density

Chl *a* content and zooxanthellae density were determined with a subset of the incubated *Acropora* sp. and *Stylophora* sp. fragments (n=4). Coral tissue was removed from the skeleton using an air-brush system with 0.2µm filtered seawater. The tissue slurry (50 ml) was homogenized and a subsample of 5 ml was collected and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the pellet was stored frozen overnight before extraction with 10 mL 90% acetone for 12 h at 4˚C in the dark. Finally, fluorescence of the sample was measured using a Trilogy Fluorometer fitted with the calibrated chl *a* non-acidification module (Turner Designs), and the calculated chl *a* content was normalized to coral surface area (µg chl *a* cm^{-2}). Another subsample (9 mL) of the tissue slurry was collected and immediately fixed in 3 mL 16% formaldehyde solution (final concentration 4%) and preserved at 4˚C for zooxanthellae density analysis. Prior to zooxanthellae density analysis the solution was centrifuged at 5000 rpm for 5 min and the pellet resuspended in a known volume of 0.2 µm filtered seawater. Subsequently, the number of zooxanthellae was counted using an improved Neubauer haemocytometer and the calculated total number of zooxanthellae in the initial tissue slurry was normalized to coral surface area (cells cm^{-2}).

Stable isotope analysis

Acropora sp. and Stylophora sp. colonies (n = 12) were collected from 5, 10 and 20 m water depth using SCUBA and immediately frozen at -20°C until further analysis. Coral tissue was removed from the skeleton using an air-brush system with 0.2µm filtered seawater. The resulting slurry was homogenized before host tissue and algal symbionts were separated in several centrifugation steps according to Muscatine et al. (1989). The zooxanthellae pellet was transferred to Eppendorf tubes and the supernatant was filtered on a pre-combusted GF/F filter (0.7 µm pore size). All samples were finally dried at 45°C for further isotopic analysis. N was analyzed for $\delta^{15}N$

signatures using a continuous flow-isotope ratio mass spectrometer. $\delta^{15}N$ values in units per mil (‰) are reported relative to the atmospheric standards and are calculated according to:

$$\delta^{15}N\ (‰) = [\ (R_{sample}/R_{standard}) - 1] * 1000$$

$$\text{and } R = {}^{15}N/{}^{14}N$$

Statistical analysis

All statistical analyses were carried out using the Statistica software package (StatSoft GmbH, Hamburg, Germany). Kruskal-Wallis ANOVA's followed by Mann-Whitney tests for Post-hoc analyses were used to analyze differences of the response parameters (P_{gross}, N_2 fixation, zooxanthellae density, chl *a* content, $\delta^{15}N$ signature) from samples collected from three different water depth (5, 10 and 20 m). Differences between day and night N_2 fixation were analyzed using a two-tailed, two-sample t-test for *Acropora* sp. and *Stylophora* sp, respectively. Finally, correlations between N_2 fixation and P_{gross} rates were determined *via* linear regression. All values are presented as mean ± SE.

Results

Gross photosynthesis and acetylene reduction

P_{gross} rates of the hard coral *Acropora* sp decreased slightly with increasing water depth, albeit not significantly, while *Stylophora* sp. revealed similar P_{gross} rates across all three water depths (Fig. 7.1A). Rates of acetylene reduction showed no significant effect of water depth in either of the two investigated hard coral genera, although both revealed a trend of decreasing acetylene reduction with increasing water depth (Fig. 7.1B). Both coral genera exhibited significantly higher (up to 3.4 times) acetylene reduction rates during day than during night (Table 7.1). Linear regression analysis between P_{gross} and N_2 fixation rates showed neither a correlation for *Acropora* sp. (F = 0.523, r^2 0.030, p = 0.480) nor for *Stylophora* sp. (F = 0.081, r^2 = 0.006, p = 0.780).

Chlorophyll a, zooxanthellae and $\delta^{15}N$ signatures

Zooxanthellae density was significantly higher in *Acropora* sp. colonies collected from 20 m water depth compared to colonies from 5 m water depth, while no significant differences were found across depths for *Stylophora* sp. corals (Fig. 7.1C). Concomitantly, the chl *a* content of both hard coral genera increased with increasing water depth, albeit significant differences were only evident for *Acropora* sp. corals (Fig. 7.1D).

The $\delta^{15}N_{Zoox}$ and $\delta^{15}N_{Animal}$ signatures of *Acropora* sp. and *Stylophora* sp. coral colonies were affected by water depth and significantly decreased along the depth gradient. Compared to $\delta^{15}N_{Animal}$, the $\delta^{15}N_{Zoox}$ values of both coral genera revealed a higher variability along the depth gradient significantly decreasing from 2.10 ± 0.35 ‰ at 5 m to 0.58 ± 0.08 ‰ at 20 m in *Stylophora* sp. corals and from 1.98 ± 0.28 ‰ at 5 m to 1.01 ± 0.06 ‰ at 20 m in *Acropora* sp. corals (Fig. 7.1E). In contrast, $\delta^{15}N_{Animal}$ values were less variable but still significantly decreased from 1.17 ± 0.13 ‰ at 5 m to 0.71 ± 0.13 ‰ at 20 m in *Stylophora* sp. corals and from 1.68 ± 0.13 ‰ at 5 m to 1.30 ± 0.09 ‰ at 20 m in *Acropora* sp. corals (Fig. 7.1F).

Table 7.1. Acetylene reduction rates (mean ± SE, nmol C_2H_4 cm^{-2} h^{-1}) of *Acropora* sp. and *Stylophora* sp. corals from three different water depths (5, 10 and 20 m) during day time and night time (D/N represents the day to night ratio of acetylene reduction rates). Significant differences (two-tailed, two-sample t-test) between day and night acetylene reduction rates are indicated by asterisk at significant levels of * < 0.05, ** < 0.005, *** < 0.001.

Primary producer	**Depth**	**Acetylene reduction rates**			**D/N**
		Day		**Night**	
Acropora sp.	5	0.24 ± 0.04	**	0.07 ± 0.02	3.4
	10	0.25 ± 0.03	*	0.15 ± 0.02	1.7
	20	0.22 ± 0.02		0.14 ± 0.03	1.5
Stylophora sp.	5	0.41 ± 0.15		0.29 ± 0.08	1.4
	10	0.34 ± 0.06		0.24 ± 0.05	1.5
	20	0.18 ± 0.02	**	0.06 ± 0.01	3.0

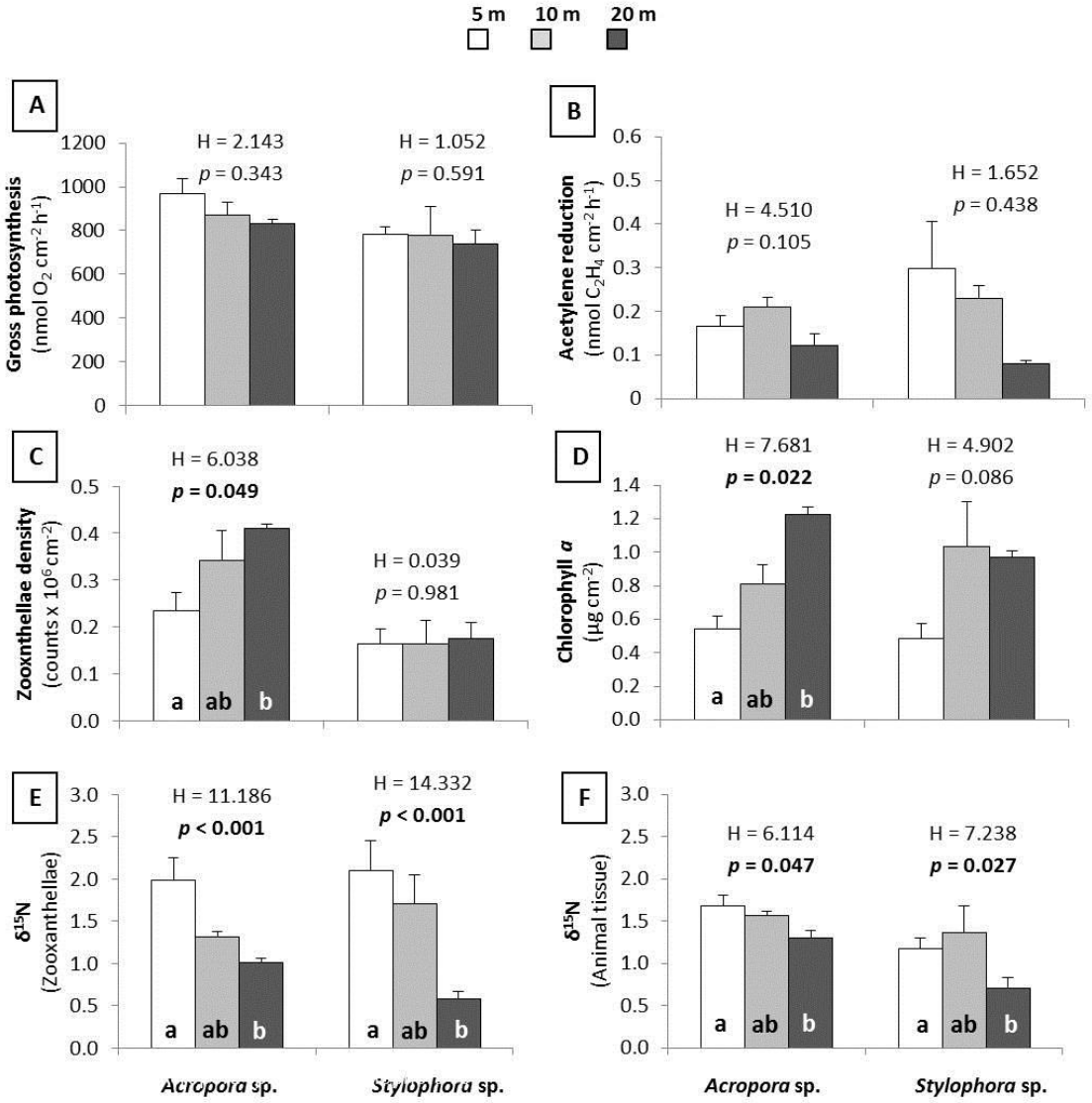

Figure 7.1. Gross photosynthesis (A, nmol O_2 cm^{-2} h^{-1}), acetylene reduction rates (B, nmol C_2H_4 cm^{-2} h^{-1}), zooxanthellae density (C), Chl a content (D), $\delta^{15}N$ signature of zooxanthellae (E) and $\delta^{15}N$ signature of the animal tissue (F) measured in *Acropora* sp. and *Stylophora* sp. corals collected from three different water depths (5, 10 and 20 m). Values are presented as mean ± SE (n = 7-8). Different letters (a-b) indicate significant differences between the three depths for each primary producer respectively, based on multiple comparisons of means (Kruskal-Wallis ANOVA).

Discussion

In the present study, the N_2 fixation activity of coral-associated diazotrophs showed no response to depth-mediated changes in daylight availability, but consistently fixed more N_2 during daytime than at nighttime. High N_2 fixation during the day indicates the involvement of heterotrophic bacteria and heterocystous cyanobacteria as both are able to avoid inactivation of nitrogenase at elevated O_2 levels (Stal 2009). A diverse group of N_2 fixing bacteria, including heterotrophic and phototrophic bacteria, has been identified in sclerectinian corals (Rohwer et al. 2002, Lesser et al. 2004, Wegley et al. 2007, Olson et al. 2009, Lema et al. 2012, Olson and Lesser 2013). Olson et al. (2009) found that heterotrophic γ-proteobacteria comprise over half the N_2 fixing bacterial community associated with *Monitopra* sp. corals. Heterotrophic bacteria may utilize the photosynthetic products from the zooxanthellae as an energy source for the energy-demanding process of N_2 fixation (i.e. the breakage of the N_2 triple bond). Lesser et al. (2007) also suggested that the endosymbiotic cyanobacteria associated with the coral *Monastraea cavernosa* are operating heterotrophically and receive the energy and reductant required for N_2 fixation from respiring glycerol. Glycerol is the main form in which C is translocated from the zooxanthellae to the host tissues (Muscatine 1990). Therefore, within the microenvironment of a zooxanthellate coral, endosymbiotic diazotrophs are likely exposed to relatively high glycerol concentrations during the day and this may be particularly advantageous for a community dominated by heterotrophic bacteria or heterocyst-forming cyanobacteria (Zehr et al. 2011). We suggest that the constant P_{gross} rates of the two investigated photo-adapted corals along the depth gradient (i.e. increased zooxanthellae densities and chl *a* content) potentially provide a stable organic C reserve. This in turn may be necessary to maintain N_2 fixation activity by the coral-associated diazotrophs under the different light availabilities.

Diazotrophs benefit from the C fixed by the zooxanthellae, but also support the functioning of the coral-zooxanthellae symbiosis in oligotrophic waters by providing an additional N source to the generally N-limited zooxanthellae (Falkowski et al. 1993, Lesser et al. 2004, 2007, Olson et al. 2009, Fiore et al. 2010). Previous studies found a significant positive correlation between the abundance of N_2 fixing bacteria and the population size or division rate of endosymbiotic zooxanthellae within the coral host (Lesser et al. 2007, Olson et al. 2009). This indicates that the N_2 fixation products are directly utilized by the zooxanthellae and is further supported by depleted $\delta^{15}N$ values of zooxanthellae from corals possessing symbiotic diazotrophs (-1.0 ‰) compared to corals (2.0 ‰) that do not (Lesser et al. 2007). Changes in the $\delta^{15}N$ signatures of coral tissue and algal symbionts can be used as indicators to determine the source and

fate of fixed N inside an organism. In the present study, corals collected from 20 m water depth revealed significantly depleted $\delta^{15}N$ values of zooxanthellae and animal tissue compared to corals from 5 m water depth. These findings are consistent with a previous study reporting increasingly depleted $\delta^{15}N$ values with increasing water depth in both the zooxanthellae (0.8 ‰ at 2 m to -1.73 ‰ at 45 m) and animal tissue (1.75 ‰ at 5 m to 0.71 ‰ at 60 m) of *Stylophora pistillata* corals from the Gulf of Aqaba (Alamaru et al. 2009). We expected that the depleted $\delta^{15}N$ values in corals growing under lower light regimes may result from increased N_2 fixation activities, although higher rates do not necessarily imply higher usage of the fixed N products. However, the corals in the present study revealed constant N_2 fixation rates under the different light regimes suggesting that corals from greater water depth may have assimilated and incorporated more of the N fixed by the diazotrophs. A potential higher N usage may support the corals' adaptations to low light conditions, such as increased photosynthetic pigment concentration and algal standing stock, both of which maximize light capture and sustain the autotrophic performance of the coral symbiosis (Dubinsky et al. 1984, Mass et al. 2007, Lesser et al. 2010). Overall, our data suggest a strong physiological relationship between photosynthesis by the zooxanthellae and N_2 fixation by the diazotrophs. A mutual interplay and trade-off of C and N_2 fixation products between zooxanthellae and diazotrophs may provide the baseline for the functioning of the coral symbiosis and its ability to adapt and thrive over a wide bathymetric range.

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Chapter VIII

Thermal stress impacts all players of the coral-algal-prokaryote symbiosis affecting the resilience of the holobiont to warming*

Abstract

Different coral holobionts (i.e., the coral-algal-prokaryote symbiosis) exhibit different thermal sensitivities which may determine if they will adapt to global warming. Thus, it is paramount to understand the role that each member plays in influencing the response of the holobiont to thermal stress. In this study, we show that exposure to higher temperature affects the main physiological traits of all partners (herein: animal host, *Symbiodinium* and diazotrophs) of both *Acropora hemprichii* and *Stylophora pistillata* during and after thermal stress. *S. pistillata* experienced severe loss of *Symbiodinium* (i.e., bleaching), while *A. hemprichii* was more resilient to thermal stress. Increased temperatures (+6 °C) resulted in decreased photosynthesis coupled with drastic increases in light N_2 fixation rates, particularly in *A. hemprichii* (by over 200 % compared to controls). After the stress event, diazotrophs exhibited a reversed diel pattern of activity, with increased N_2 fixation rates recorded only in the dark (by over 100 % compared to controls). Concurrently, both animal hosts displayed impaired organic matter release and picoplankton feeding. These results demonstrate that thermal stress impacts all holobiont members, and suggest that physiological plasticity of coral-associated diazotrophs, by providing additional nitrogen under high temperature, may play a key role during bleaching events.

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Introduction

Scleractinian corals are the fundament of tropical reef ecosystems. These animals owe their high productivity and growth to the symbiosis with dinoflagellate photosynthetic algae of the genus *Symbiodinium*, which provide the coral host with essential nutrients and sugars to sustain their energy requirements (Dubinsky & Jokiel 1994). However, thermal stress can cause the breakdown of the coral-algal symbiosis, with the loss of *Symbiodinium* (i.e., coral bleaching) having severe consequences for the fitness and survival of the coral host (Brown 1997). Mass bleaching events are predicted to occur more frequently as a result of global warming, and can result in high coral mortality over wide areas, threatening the stability of coral reef ecosystems worldwide (Hughes et al. 2003).

Given that primary productivity in tropical reef environments is typically nutrient limited (Atkinson 2011), nutrients have a key role in determining coral resistance and resilience to thermal stress. *Symbiodinium* densities generally increase under nutrient replete conditions (Fabricius 2005, Stambler et al. 1991) and the addition of nutrients cause a reduced coral susceptibility to seasonal bleaching (McClanahan et al. 2003). However, nutrient enrichment can also make corals more vulnerable to thermal stress (Vega Thurber et al. 2014), especially when the essential nutrients (mainly nitrogen and phosphorous) are not available to *Symbiodinium* at sufficient concentrations to ensure a chemically balanced growth (Wiedenmann et al. 2013).

In the absence of *Symbiodinium*, corals are deprived of their main energy producers and must rely on alternative sources to meet their metabolic needs. For instance, bleached corals can adapt and acquire more energy through heterotrophic feeding (Grottoli et al. 2006, Palardy et al. 2008). Still, different coral species have different capabilities for heterotrophic plasticity (Grottoli et al. 2006), and other pathways of nutrient acquisition may be very important to determine coral sensitivity to thermal stress.

Besides their symbiotic algae, corals are associated with a vast diversity of microorganisms. It is increasingly recognized that it is the functioning of the association among all the partners (i.e., coral holobiont) that determines the success of hard corals as the primary ecosystem engineers of tropical coral reefs (Barrot & Rohwer 2012). Over the last decade, a vast number of studies characterized the diversity of microbial communities in corals by using molecular tools. Several of these studies demonstrated that coral-associated microbial communities are usually stable, until an event of external origin (e.g., changes in environmental conditions) impairs the functional stability of the partnership causing pathogenic microbes to proliferate, or beneficial microbes to become

detrimental to the coral host (Bourne et al. 2007, Littman et al. 2011). As such, stable microbial communities found in heathy corals promote their health (Krediet et al. 2013, Rosenberg et al. 2007). Nevertheless, we still have a limited understanding of the roles of these beneficial bacteria, which likely provide functional stability to the partnership.

Recently, several studies found N_2-fixing bacteria (i.e., diazotrophs) to be common partners in the coral holobiont Lesser et al. 2004, 2007, Lema et al. 2012, 2014a, 2014b, Olson et al. 2009). Diazotrophic assemblages in corals are consistent among different reef locations, are species-specific, and establish in the early life history stages of their coral host (Lema et al. 2012, 2014a, 2014b). Potentially, these bacteria are functionally very important in oligotrophic tropical environments, given their unrivalled capability of fixing molecular nitrogen (N_2) and making it available to the holobiont. More importantly, the association with diazotrophs may be fundamental to the functioning of the holobiont under stressful conditions, such as during periods of increased temperatures (Cardini et al. 2014).

Here, we investigate the interactions between two common hard coral hosts from the northern Red Sea, *Acropora hemprichii* and *Stylophora pistillata*, and their associated algae as well as diazotrophs, exposing the coral holobiont to a thermal stress experiment lasting three weeks. The goals of this study were to characterize, quantify and compare the main ecological traits of the coral host (feeding, calcification, organic matter release), of the associated *Symbiodinium* (primary productivity) and of the associated diazotrophs (N_2 fixation) in response to thermal stress. Importantly, our study setting is characterized by corals with exceptionally high bleaching thresholds, and it has thus been suggested to serve as reef refugium from global warming (Fine et al. 2013). Here, we used a comparative approach among two coral species with different bleaching susceptibilities, making it possible to assess the physiological interactions among the remaining partners once the photosynthetic algae is lost from the association (i.e., bleaching occurs). These data provide the capacity to link the different functional roles of the partners of the coral holobiont to better evaluate their significance in determining the response of corals to the increased seawater temperatures resulting from global warming.

Methods

Sample collection and maintenance

Acropora hemprichii and *Stylophora pistillata* nubbins (4-5 cm in height) were collected haphazardly at 10 m water depth from coral colonies along the slope of the fringing reef in front of the Marine Science Station (MSS) Aqaba, Jordan (29° 27' N, 34° 58' E) in September 2013. Branches of 6-8 cm in height were cut from mother colonies which were at least 5 m apart using bone cutters, placed in plastic bags and transported back to the laboratory taking care not to cause any abrasion of the tissues. A total of 32 fragments were subsequently glued on ceramic tiles and allowed to recover for two weeks before starting the manipulation experiment. Corals were maintained in tanks directly supplied with untreated reef water (from 10 m water depth) and covered with layers of black mesh to simulate natural levels of light intensity at the sampling location. Temperature and light in the aquaria were monitored using HOBO data loggers (Onset HOBO Pendant UA-002-64; Bourne, MA, USA). Measurements of light intensity were also performed using a quantum sensor (Model LI-192SA; Li-Cor, Lincoln, Nebraska, USA) parallel to data loggers to allow conversion from light intensity (LUX) to photosynthetically active radiation (PAR, μmol photons m^{-2} s^{-1}, wavelength 400 to 700 nm). Inorganic nutrient concentrations of the reef water were analyzed once per week. For each inorganic nutrient analysis, samples of 50 mL were collected (n = 4) and gently filtered through cellulose acetate membrane filters (nominal pore size: 0.45 μm). Immediately after filtration, inorganic nutrient concentrations (ammonium - NH_4^+, phosphate - PO_4^{3-}, nitrate - NO_3^-, nitrite - NO_2^-) were measured using established standard methods (Murphy & Riley 1962, Strickland & Parsons 1972, Holmes et al. 1999). NH_4^+ was determined fluorometrically using a Trilogy fluorometer (Turner Designs), while all other inorganic nutrients were measured spectrophotometrically with a V-630 UV-Vis Spectrophotometer (Jasco Analytical Instruments). Detection limits for NH_4^+, PO_4^{3-}, NO_x and NO_2^- were 0.09, 0.01, 0.02 and 0.005 μM, respectively. Environmental variables during the experiment other than temperature are reported in the supplementary material, Table 8.S1.

Thermal stress experiment

We tested the response of *Stylophora pistillata* and *Acropora hemprichii* to thermal stress by exposing coral nubbins to increasing water temperatures at a rate of 1 °C every two days (Figure 8.1). Temperature changes were made to correspond with daily natural peak temperature (14.30 - 16.30). Once at 31.3 ± 0.6 °C, corals were kept at constant temperature for 4 days before exposing

them to a peak of 34.3 ± 0.3 °C for 5 h. Subsequently, temperature was decreased at a rate of 2 °C per day until it reached the control temperature again. Coral nubbins were exposed to the manipulation treatment in a tank directly supplied with untreated reef water next to the flow-through aquarium where control nubbins were maintained. Control nubbins received seawater with no temperature manipulation (temperature throughout the experiment = 24.9 ± 0.4 °C). Temperature and light in the tanks were monitored using HOBO data loggers. Incubation experiments took place at the following sampling times: day 0 (2 days prior to the start of the manipulation treatment), day 13 (at which point treated nubbins had been exposed to 31 °C for 36 h), and day 21 (in which the treatment was back to control temperature since 48 h). Net photosynthesis (P_N), dark respiration (R_D), N_2 fixation and POC and DOC release of the coral nubbins were measured in their respective treatment tank to ensure maintenance of stable temperature in the chambers. Immediately prior to the start of the incubations, ceramic tiles were cleaned from sediment and epibionts with a fine brush. Bleached nubbins had polyps open at night, and only nubbins without any sign of tissue loss were used in the incubation experiments. Incubation water was taken from the respective treatment tank, and nubbins were allowed to adjust to chamber conditions for at least 15 min prior the start of the incubations. A stir bar powered by a submersible magnetic stirrer (600 rpm, Cimarec i Telesystem Multipoint Stirrers, Thermo Scientific) ensured water mixing (and gas equilibration with the headspace, if applicable) in the chambers. A data logger was placed in an additional chamber to monitor temperature and light intensity during the incubations. Dissolved oxygen (DO) concentration was measured in each chamber following the incubations to ensure that oxic conditions were maintained. All physiological parameters were normalized to skeletal surface area of the coral nubbins, measured using the Advanced Geometry protocol (Naumann et al. 2009).

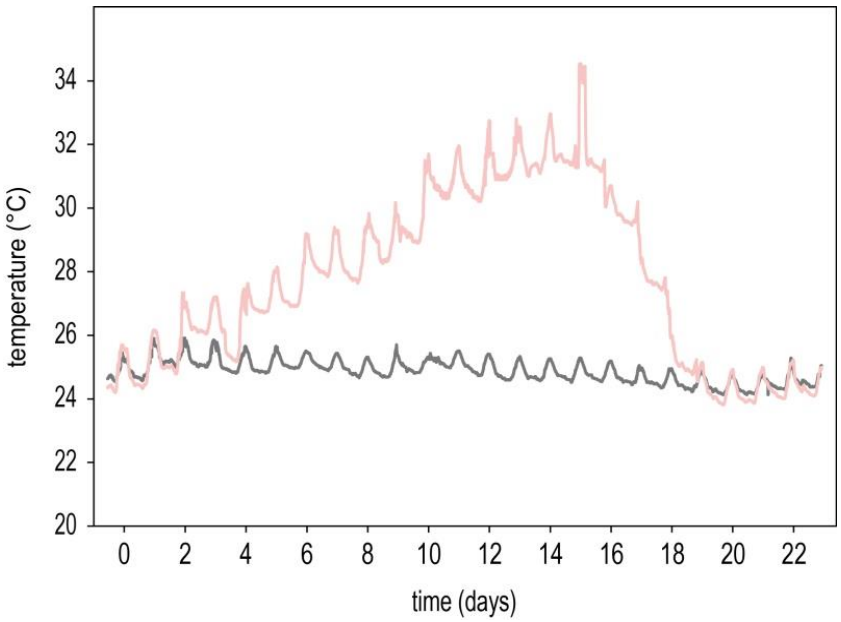

Figure 8.1. Temperature profiles of the two thermal treatments with which *Stylophora pistillata* and *Acropora hemprichii* nubbins were challenged. In the treatment (red line), corals were exposed to increasing temperatures up to a maximum of 34 °C, while control corals (grey line) were kept at *in-situ* temperature.

Symbiodinium density and chlorophyll a content

At the end of the experiment, tissue was removed from the skeleton of the coral nubbins using a jet of pressurized air and 0.2 µm-filtered seawater. The resulting tissue slurry (50 mL) was homogenized using a vortex, and one aliquot of 9 mL was subsequently subsampled, fixed in 3 mL 16 % formaldehyde solution (final concentration 4 %) and preserved at 4 °C until *Symbiodinium* density analysis. The homogenate was subsequently centrifuged at 5000 rpm for 5 min, the supernatant removed, and the pellet resuspended with a known volume of 0.2 µm-filtered seawater. The number of zooxanthellae was counted using an improved Neubauer haemocytometer (pillay et al. 2009).

A second aliquot of 5 mL was taken from each homogenate, immediately centrifuged at 5000 rpm for 5 min, and the supernatant discarded. The pellet was frozen at -20 °C overnight to break the cells and subsequently resuspended in 10 mL 90 % acetone for 24 h in the dark at 4 °C. After another centrifugation run, chlorophyll *a* was measured using the non-acidification fluorometric method (Welschmeyer 1994) on a Trilogy fluorometer equipped with the non-

acidification chl module (CHL NA #046). Visual analyses of coral color according to the international Reef Check guidelines were also performed to monitor coral health during the entire duration of the experiment.

Symbiodinium primary productivity

Net photosynthesis (P_N) and dark respiration (R_D) were assessed measuring DO fluxes in closed-cell respirometric glass chambers (1 L). P_N was measured during the most stable light conditions of the day (12:00 - 14:00), and R_D was measured in the dark. DO fluxes were assessed as differences between salinity corrected DO concentrations (incubation time ≈ 90 min) measured with an DO optode sensor (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). Gross photosynthesis (P_G) was calculated ($P_G = P_N + R_D$) for each specimen, and should be considered a conservative estimate. The $P_N : R_D$ ratio was also calculated to determine the effect of thermal stress on coral metabolism. Unfiltered seawater and unfiltered seawater plus the mounting tile were also incubated as controls ($n = 8$). Differences in DO concentration were subsequently corrected for control signals and normalized to incubation period and nubbins surface area.

Coral calcification

Coral light calcification was determined during the incubation for P_N using the alkalinity anomaly technique (Chisholm & Gattuso 1991). Samples (50 mL) for total alkalinity were collected from each chamber at the beginning and end of the incubations and immediately filtered through 0.45 µm cellulose acetate membrane filters into falcon tubes. Samples were subsequently poisoned with 0.04 % of a 50 % mercury chloride solution and preserved at 4 °C in the dark until measurement. Total alkalinity was determined by open cell potentiometric titration with a TitroLine alpha 05 plus (SI Analytics). The analytical precision of the measurements was determined with three standards across each measurement run (0.5 N Na_2CO_3 alkalinity standard ampoules (HACH 14278-10) diluted to 2500 µmol kg^{-1}) and was less than ± 5 µmol kg^{-1}. Changes in inorganic nutrient concentrations can also affect total alkalinity (Wolf-Gladrow et al. 2007). Therefore, samples for phosphate and ammonium concentrations were taken from the coral chambers, but no measurable differences were detected during the incubations. Calcification rates were finally normalized to incubation period and nubbins surface area.

Coral mucus release

Coral mucus release was quantified during each of the four seasons using the established beaker incubation method (Wild et al. 2005, Naumann et al. 2010). Coral nubbins and unfiltered seawater controls were incubated for 6 h during the day (10:00 – 16:00 h) in open chambers (1 L) covered with transparent cellophane to prevent the input of airborne particles, leaving small side openings for air exchange. Chambers were not stirred to allow for comparisons with previous studies (Naumann et al. 2010) and to rule out the influence of water currents on mucus release and fractionation. Samples for dissolved organic carbon (DOC) and particulate organic carbon (POC) were collected at the start and end of the 6 h incubation period. Initial samples (n = 3) for POC were collected from the ambient aquarium water, while simultaneously filling the chambers. At the end of the incubation, coral nubbins were carefully removed from the incubation chambers with clean tweezers, and the remaining incubation water was filtered for POC, after collecting water samples for measurements of DOC, picoplankton concentrations and microbial respiration of released coral mucus (see respective method section). The water volume was measured and vacuum-filtered onto pre-combusted (450 °C, 4h) GF/F filter (VWR, diameter: 25 mm, nominal pore size 0.7 µm). Filters were dried for 48 h at 40 °C, and stored dry until analysis. POC filters were acidified (0.1N HCl) prior to analysis, and carbon content of the dried filters was measured using a EuroVector elemental analyzer (EURO EA 3000). Carbon contents were derived from calculations using elemental standards (OAS 187560; analytical precision ≤0.1% (C) and ≤0.03% (N) of the standard value). At the beginning and end of the incubation, samples for DOC (50 mL) were collected with an acid-washed 50 mL polycarbonate syringe from each chamber and gently vacuum-filtered (max. suction pressure 20 kPa) through a pre-combusted GF/F filter directly into acid-washed 30 mL HDPE sample bottles using a custom set-up with an in-line polycarbonate filter holder. Prior to filtration, the syringes, the HDPE sample bottles and the filtration set-up were soaked in 0.4M HCl for 24 h and then washed with MQ water. Upon collection, the first 20 mL of sample was used to wash the filtration set-up and the sample bottle with 2 x 10 mL sample water and discarded before collecting the remaining 30 mL. DOC samples were immediately acidified with 80 µl of 18.5 % HCl and stored in the dark at 4 °C until analysis. Samples were analyzed by the high-temperature catalytic oxidation (HTCO) method on a Shimadzu TOC-V_{CPH} total organic carbon analyzer. The instrument was calibrated with a 10-point calibration curve of serial dilutions from a potassium hydrogen phthalate certified stock solution (1000 ppm Standard Fluka 76067-500ML-F). Consensus reference material provided by DA Hansell and W Chan of the University of Miami (Batch 13, Lot #08-13, 41-45 µmol C L^{-1}) was used as positive control between every 10 samples. Each sample was measured with 5 replicate injections, and analytical precision was < 3 %

of the certified value. For the calculation of mucus release rates (POC and DOC), concentration differences measured between the incubation start and end of each coral chamber were corrected by the average control signal (n = 6) and normalized by the incubation volume, incubation period and coral surface area. Total organic C (TOC) release was calculated as the sum of POC and DOC release for each coral nubbin.

Coral picoplankton feeding

Flow cytometry samples for quantification of picoplankton feeding were collected at the beginning and end of the coral mucus release incubations. Initial samples (n = 3) were collected from the ambient aquarium water, while simultaneously filling the chambers. Final samples were collected from each chamber (treated nubbins, control nubbins and seawater without nubbins) at the end of the incubations. Seawater controls were used to estimate the autogenic changes in picoplankton concentrations resulting from internal grazing, natural death or growth. Samples (2 mL) for flow cytometry analysis were fixed with 0.1 % paraformaldehyde (final concentration) for 30 min at room temperature, frozen with liquid nitrogen, and stored at -80 ^{0}C until analysis. *Synechococcus* sp., *Prochlorococcus* sp., and picoeukaryotes were quantified with a flow cytometer (FACSCalibur, Becton Dickinson, 488 nm excitation laser). *Synechococcus* sp., *Prochlorococcus* sp., and picoeukaryotes were analyzed at a flow rate of ~ 0.06 µl min^{-1} for 2 min and gated on a dot plot of orange fluorescence versus red fluorescence using the program CellQuestPro. The instrument flow rate was calibrated gravimetrically according to Current Protocols in Cytometry (citation). Growth rates of picoeukaryotes and cyanobacteria (*Synechococcus* sp. + *Prochlorococcus* sp.) and coral grazing on picoeukaryotes (expressed as removal rate) were subsequently calculated using the equations from (Houlbrèque et al. 2004).

Microbial coral-associated N_2 fixation

N_2 fixation was quantified by using an adapted acetylene (C_2H_2) reduction technique (Capone 1993, Wilson et al. 2012). 1 L glass chambers were filled with 800 mL of natural seawater, whereof 10 % was replaced with C_2H_2-saturated seawater. The chamber was immediately sealed gas-tight and 10 % of the 200 mL headspace was replaced with C_2H_2 gas through a gas-tight rubber stopper equipped with a push-button syringe valve with Luer lock (SGE Analytical Science). C_2H_2 and the C_2H_2-saturated seawater were freshly prepared ≈10 min prior to use. N_2 fixation

incubations lasted for 6 h and were repeated during the night (23:00 - 5:00) and during the day (11:00 - 17:00) to quantify dark and light N_2 fixation rates. Gas samples (1 mL) were withdrawn from the headspace after time intervals of 0, 1, 6 h and collected in glass vials with grey butyl stoppers (CS-Chromatographie Service GmbH) previously filled with deionized water. Vials were stored frozen upside-down until analysis to prevent any leakage via the septa. Prior to analysis, vials were thawed, and water and gas phases in the vials were equilibrated at room temperature by vigorous shaking. Ethylene (C_2H_4) concentration in the vials' headspace was measured using a reducing compound photometer (RCP; Peak Laboratories) fitted with a 10 μL sample loop (accuracy, ± 100 ppb). The instrument was calibrated using serial dilutions of a 200 ppm (± 2 %) C_2H_4 standard in air (Restek, Bellefonte, PA, USA). Differences in C_2H_4 concentration between the time intervals of the incubation period were converted into hourly C_2H_4 evolution rates according to Breitbarth, *et al.* (Breitbarth et al. 2004). C_2H_4 concentrations were corrected for the signal of unfiltered seawater controls (n = 8) and normalized to incubation time and surface area of the specimens in order to calculate C_2H_4 production rates. Additional controls for 0.2 μm filtered seawater (n = 6), unfiltered seawater and ceramic tile (n = 6), and unfiltered seawater with substrate samples, but no addition of C_2H_2 (natural C_2H_4 production, n = 6), showed negligible C_2H_4 production. Finally, a 3:1 molar ratio of C_2H_4:N_2 was used to estimate the nitrogen flux to the holobiont (Lesser et al. 2007).

Microbial respiration of released coral mucus

To measure utilization of coral mucus by the planktonic microbial community in treated and control coral nubbins, samples of 60 mL were collected from all incubation chambers at the end of the coral mucus release incubations, after homogenizing the water volume. The remaining subsamples were transferred to ground-glass stoppered bottles (Wheaton BOD), and initial oxygen concentration of each subsample was determined with a DO optode sensor (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). Samples were kept airtight in the dark at *in situ* temperature. After 24 h, DO values were measured again to assess microbially mediated oxygen fluxes. Hourly differences in DO concentrations were corrected by the average control signal (n = 6) and normalized by the mucus release incubation period, chamber volume and coral surface area.

Statistical analysis

Differences in each parameter were assessed using univariate distance-based permutational nonparametric analyses of variance (PERMANOVA) (Anderson 2001). A design with three fixed factors (species, treatment, time) was used to test for differences in P_N : R_D ratio, P_G, N_2 fixation, calcification and mucus release rates. Concurrently, a design with two fixed factors was used to test for differences in *Symbiodinium* density, areal and *Symbiodinium* chlorophyll *a* content, picoeukaryotes and cyanobacteria growth, picoeukaryotes removal and microbial oxygen consumption between species and treatments. Datasets with negative values were made positive by constant addition, and absolute values were used in the case of R_D. Subsequently, data were square root transformed, and analyses were based on Bray Curtis similarities. As sample size was in some cases low or unbalanced due to methodological reasons, type III (partial) sum of squares was used with 9999 unrestricted permutations of raw data, and pair-wise tests were carried out if significant differences occurred ($p < 0.05$). We did not use repeated-measures analysis, because it assumes that repeated measurements are made on the same individual experimental units. During our experiment, we measured a range of physiological parameters many of which depend on the microbial and algal community abundance and composition. *Symbiodinium* density changed during the course of the experiment, and the microbial community likely changed as well, and we therefore could not assume that our experimental units were the same entities. PERMANOVA tests were performed using the homonymous routine included in the software PRIMER 6+.

Results

Thermal response: Symbiodinium-mediated processes

The two coral species responded differently to the thermal stress experiment. *Stylophora pistillata* suffered significant bleaching, and *Symbiodinium* density and areal chlorophyll *a* content decreased by more than 90 % in the treatment compared to the controls ($p < 0.05$ and $p < 0.001$, respectively, PERMANOVA with pair-wise comparisons; Figure 8.2*a*,*b*). *Symbiodinium* cell chlorophyll *a* content also significantly decreased ($p < 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.2*c*). Conversely, *Acropora hemprichii* did not show any significant loss neither of *Symbiodinium* nor of photopigments ($p > 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.2*a*,*b*,*c*).

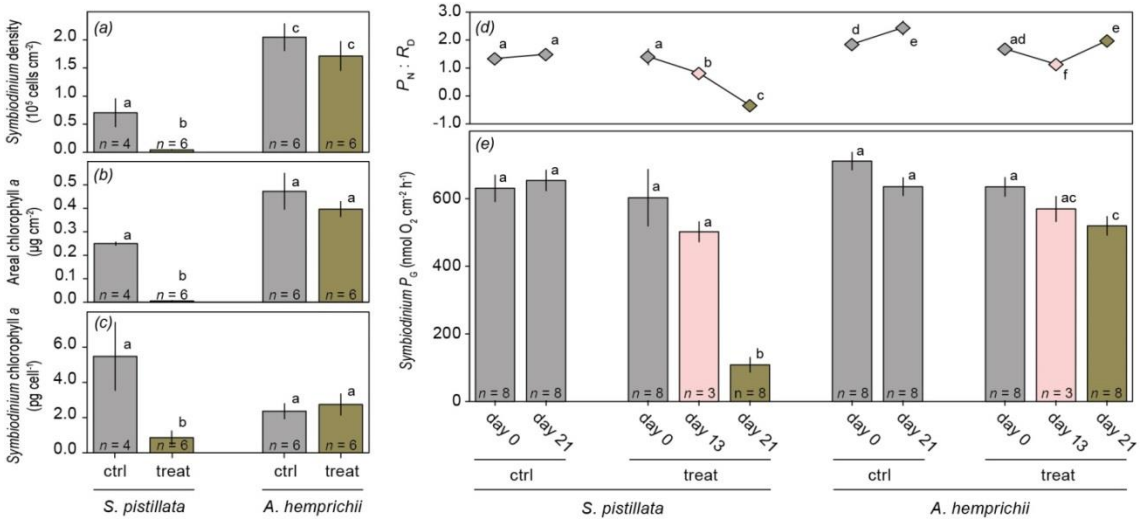

Figure 8.2. *Symbiodinium*-mediated processes. ***(a)*** *Symbiodinium* density (cells cm^{-2}), ***(b)*** areal chlorophyll *a* (µg cm^{-2}) and ***(c)*** *Symbiodinium* chlorophyll *a* (pg cell^{-1}) in the coral nubbins at the end of the experiment. ***(d)*** Net photosynthesis versus dark respiration (P_N : R_D) and ***(e)*** gross photosynthesis (P_G, nmol O_2 cm^{-2} h^{-1}) of the coral nubbins measured at different times during the experiment. Values are means ± s.e. (*n* for each group indicated, with P_N : R_D having the same *n* as P_G) and different letters indicate a significant full interaction term ($p < 0.05$, PERMANOVA with pairwise tests). Grey bars, ambient temperature; pink bars, 31 °C exposure; green bars, ambient temperature after exposure to 34 °C. Results of the PERMANOVAs are reported in the supplementary material, table 8.S2 and 8.S3.

In *S. pistillata*, the loss in *Symbiodinium* and chlorophyll *a* was triggered by the exposure of treated nubbins to the peak temperature of 34 °C, while no colour loss was observable during exposure to 31 °C. These visual observations were consistent with measurements of P_G, which remained stable in *S. pistillata* at day 13, but dropped significantly at day 21 ($p < 0.01$, PERMANOVA with pair-wise comparisons; Figure 8.2*e*). Concurrently, a significant decrease in P_N : R_D ratio was observed over the course of the experiment ($p < 0.01$, PERMANOVA with pair-wise comparisons; Figure 8.2*d*). In *A. hemprichii*, the P_N : R_D ratio decreased significantly at day 13, but increased again at the end of the experiment ($p < 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.2*d*). Conversely, P_G was significantly lower at day 21 compared to day 0 and to the controls ($p < 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.2*e*).

Thermal response: coral-mediated processes

Coral light calcification in both *S. pistillata* and *A. hemprichii* decreased at day 13 under high temperature. However, differences were deemed not significant by the statistical test ($p > 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.3*a*). At the end of the experiment, mucus release rates were lower in treated coral nubbins, particularly in *S. pistillata* (t-test, $p<0.01$, day 0 ($n = 5$) versus day 21 ($n = 8$)). However, the PERMANOVA test detected only a significant effect of the factor time ($p < 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.3*b*), whereas the interaction term was not significant. This was mainly due to the high variability in DOC release rates (supplementary material, Figure 8.S1*a* and Table 8.S2), while a significant decrease in POC release was measured in *S. pistillata* ($p < 0.001$, PERMANOVA with pair-wise comparisons; supplementary material, Figure 8.S1*b* and Table 8.S2) but not in *A. hemprichii*.

Picoeukaryotes and cyanobacteria populations had higher growth rates in incubation chambers with treated corals and in seawater controls than in chambers in which the control nubbins were incubated, indicating decreased feeding by treated corals (Figure 8.3*c,d*). However, differences were deemed not significant by the statistical test ($p > 0.05$, PERMANOVA with pair-wise comparisons). Nevertheless, picoeukaryotes removal rates were significantly lower in treated nubbins compared to control nubbins ($p < 0.001$, PERMANOVA with pair-wise comparisons; Figure 8.3*e*), confirming a negative effect of thermal stress on picoplankton feeding.

Thermal response: microbes-mediated processes

N_2 fixation by the coral-associated microbial community responded to the thermal treatment. In the light, a significant increase in N_2 fixation was detected for both corals when exposed to 31 °C ($p < 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.4*a*), while fixation rates at the end of the experiment were not different from day 0 and from the controls for both coral species. In the dark, N_2 fixation remained constant at day 13 (31 °C), while increased significantly in treated coral nubbins at the end of the experiment ($p < 0.05$, PERMANOVA with pair-wise comparisons; Figure 8.4*a*).

Concurrently, in the water surrounding treated corals, microbial respiration of released coral mucus decreased significantly ($p < 0.001$, PERMANOVA with pair-wise comparisons; Figure 8.4*b*) in response to the decreased organic matter fluxes (Figure 8.3*b*).

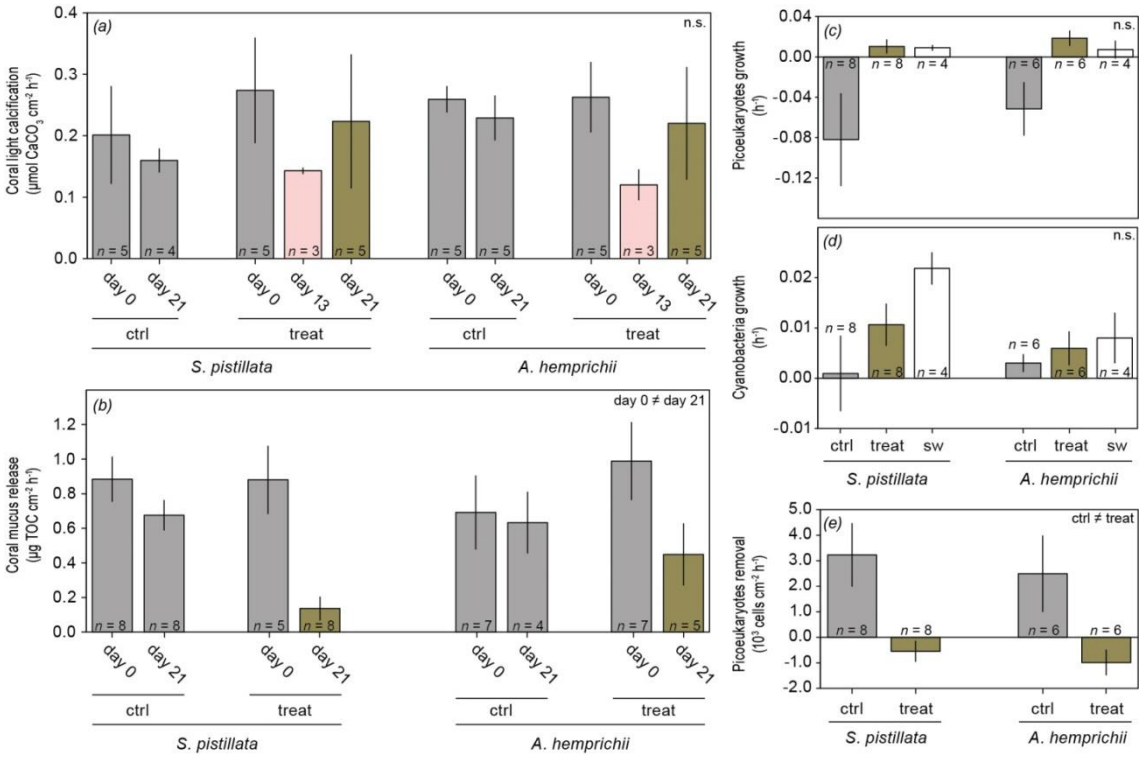

Figure 8.3. Coral-mediated processes. ***(a)*** Coral light calcification (μmol $CaCO_3$ cm^{-2} h^{-1}) and ***(b)*** mucus release (μg TOC cm^{-2} h^{-1}) at different times during the experiment. ***(c)*** Picoeukaryotes growth (h^{-1}) and ***(d)*** cyanobacteria growth (h^{-1}) in coral-surrounding water versus seawater controls and ***(e)*** picoeukaryotes removal by the corals (cells cm^{-2} h^{-1}) at the end of the experiment. Values are means ± s.e. (*n* for each group indicated) and pairwise comparisons for significant factors ($p < 0.05$, PERMANOVA) are indicated on the top right corner of each graph (n.s., not significant). Grey bars, ambient temperature; pink bars, 31 °C exposure; green bars, ambient temperature after exposure to 34 °C; white bars, ambient temperature without coral nubbins. Results of the PERMANOVAs are reported in the supplementary material, table 8.S2 and 8.S3.

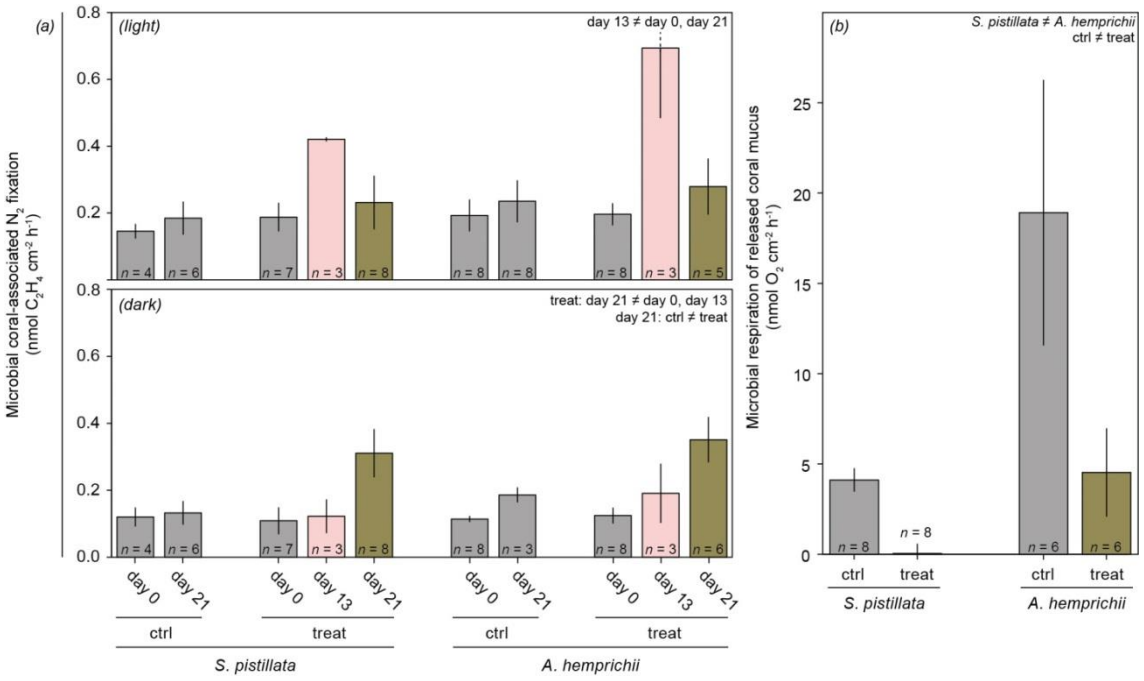

Figure 8.4. Microbe-mediated processes. ***(a)*** Light and dark microbial coral-associated N_2 fixation (nmol C_2H_4 cm^{-2} h^{-1}) at different times during the experiment. ***(b)*** Microbial respiration of released coral mucus (nmol O_2 cm^{-2} h^{-1}) in coral-surrounding water at the end of the experiment. Values are means ± s.e. (*n* for each group indicated) and pairwise comparisons for significant factors ($p < 0.05$, PERMANOVA) are indicated on the top right corner of each graph. Grey bars, ambient temperature; pink bars, 31 °C exposure; green bars, ambient temperature after exposure to 34 °C. Results of the PERMANOVAs are reported in the supplementary material, table 8.S2 and 8.S3.

Discussion

Our results demonstrate that thermal stress affects all members of the coral holobiont. Some of the main physiological parameters which determine the overall functioning of the holobiont, such as photosynthesis, mucus release, heterotrophic feeding, and N_2 fixation were all impaired by thermal challenge.

Several studies demonstrated the susceptibility of the coral-algal symbiosis to high temperatures (Jokiel & Coles 1990, Fitt et al. 2001). Consequently, coral reef ecosystems are endangered in global warming scenarios that may result in mass coral bleaching and subsequent mortality over wide reef areas (Donner et al. 2005, Hoegh-Guldberg et al. 2007). During our experiment, both species were highly resistant to thermal stress, showing no evident sign of bleaching at temperatures of 31 °C (6 °C above ambient) which were maintained for four days. These temperatures are 2 °C above the theoretical bleaching threshold for the Gulf of Aqaba (Fine et al. 2013) and are reported to cause bleaching in several coral species from the Caribbean to the Indo-Pacific (Winter et al. 1998, Marshall & Baird 2000, Manzello et al. 2007). Our study supports previous results which showed that corals from the northern Red Sea are highly resistant to thermal challenge since they originate from coral genotypes of the central Red Sea, where temperature often exceeds 32 °C (Fine et al. 2013). Thus, in the Gulf of Aqaba these corals live well below their physiological bleaching threshold (Fine et al. 2013). During our experiment, *S. pistillata* endured an almost complete loss of *Symbiodinium* after being exposed to temperatures up to 34.5 °C for 5 h, while the coral-algal symbiosis remained intact in *A. hemprichii*. These results highlight the exceptional resilience of *A. hemprichii* to thermal challenge. Concurrently, the loss of *Symbiodinium* did not result in any tissue loss in *S. pistillata*, and the coral tissue, although bleached, appeared healthy at the end of the experiment (supplementary material, Figure 8.S2) suggesting potential for recovery.

Despite the high resistance of the investigated corals to thermal challenge, the main coral-mediated processes were impacted by thermal stress. Light calcification was the least affected among the physiological processes investigated. The response of hard coral calcification to thermal stress is highly variable, with some species showing decreased calcification during bleaching (Leder et al. 1991, Rodrigues & Grottoli 2006), while others immediately after *Symbiodinium* is lost (Suzuki et al. 2003). Moreover, some species show an almost complete cessation of calcification with increased temperature, while other species are less affected (Carricart-Ganivet et al. 2012). During our study, calcification slightly decreased in the high temperature, but was similar to the controls at the end of the experiment for both corals. These results suggest that these two species are capable

of skeletal accretion even during thermal anomalies and bleaching events. However, they may lose this capability if exposed to repeated thermal stress events that reduce their fitness and recovery potential (Grottoli et al. 2014).

Heterotrophic picoplankton feeding was reduced after the thermal stress event in both coral species, regardless if the coral lost its photosymbionts or not, and treated nubbins removed a significantly lower amount of picoeukaryotes from the seawater. Heterotrophic plasticity in response to bleaching appears to be highly species-specific, with some species that are able to adapt and increase their feeding rates, while others are not (Grottoli et al. 2006, Palardy et al. 2008, Ferrier-Pagès et al. 2010, Tremblay et al. 2012). In this context, the relevance of pico- and nanoplankton feeding in corals has been highlighted in previous studies (Houlbrèque et al. 2004, Tremblay et al. 2012). Importantly, our study investigated coral feeding on picoeukaryotes and cyanobacteria under natural prey concentrations and in the light. Our results of decreased picoeukaryotes removal in treated corals stand in contrast with a previous study that found bleached *S. pistillata* to have increased pico- and nanoplankton grazing rates (Tremblay et al. 2012). However, in this other study the prey concentration was manipulated, and corals were incubated in the dark, making it difficult to compare those results with our findings. Overall, our study reveals that the feeding capacity of corals on picoplankton in the natural environment may be impacted by thermal stress events. Given the importance of heterotrophic carbon inputs when the photosynthetic apparatus of the holobiont is impaired, our results have strong implications for a better understanding of the consequences of global warming on coral resilience.

Concurrently, thermal-stressed corals decreased their release of mucus to the surrounding seawater, particularly in the case of bleached *S. pistillata*. Other authors found an increase of organic matter release in scleractinian corals in response to thermal bleaching (Niggle et al. 2009, Tremblay et al. 2012). In these studies, the increased excretion of mucus was detected at the very beginning of the heat stress, and may represent a host strategy to limit photoinhibition of *Symbiodinium* during the early stages of thermal bleaching (Wooldridge 2009, Tremblay et al. 2012). Conversely, in our study, mucus release rates were quantified when the thermal stress ceased. Thus, our results indicate that recovering corals retained their energy reserves reducing the production and regeneration of their mucus surface layer and its concomitant release as organic matter. Given the importance of coral mucus in the biogeochemical cycling and functioning of coral reef ecosystems (Wild et al. 2004), the consequences of decreased mucus release following bleaching events may be significant. These speculations are confirmed by our results of microbial respiration of released coral mucus, which decrease significantly as a result of the decreased mucus release. On an ecosystem scale, bleaching

events may therefore negatively impact the microbial regeneration of nutrients in the water column and in the reef benthos.

The functioning of the microbial community of the coral holobiont was also affected by thermal stress, as indicated by our results on N_2 fixation by coral-associated diazotrophs. While the importance of coral-associated bacteria during the onset of thermal bleaching has been established in several studies (Bourne et al. 2007, Rosenberg et al. 2009, Vega Thurber et al. 2009), little is known about how these microorganisms contribute to the functioning of the coral holobiont under stress. Concurrently, there is increasing evidence that diazotrophic bacteria may play an important role to the coral by providing bioavailable nitrogen in otherwise nutrient-depleted tropical waters (Lesser et al. 2007, Lema et al. 2012), and a recent study found coral-associated diazotrophs abundance and diversity to increase at increased temperature (Santos et al. 2014). Our results demonstrate that diazotrophic microbes may play a key role during periods of high temperatures as well as during post-stress recovery of the holobiont. Under high temperature conditions, an increase in light N_2 fixation by over 200 % as opposed to controls was detected in *A. hemprichii*, while a sharp (but minor) increase was also measured in *S. pistillata*. In *A. hemprichii*, this resulted in a nitrogen flux of 0.16 μg N cm^{-2} d^{-1}, while control corals showed an average flux of 0.05 μg N cm^{-2} d^{-1}. While these fluxes are low compared to other potential sources of nitrogen such as heterotrophic feeding (Houlbrèque & Ferrier-Pagès 2009), during a bleaching event they may be relevant. Indeed, it is increasingly recognized that the nutrient status of the holobiont impact its susceptibility to coral bleaching (Ferrier-Pagès et al. 2010, Wiedenmann et al. 2013). An imbalanced supply of nitrogen (resulting for example from terrestrial runoff) can result in phosphate starvation of the symbiotic algae, therefore having a detrimental effect on the coral resistance to thermal stress (Wiedenmann et al. 2013). Conversely, moderate nitrogen enrichment benefit corals, which show increased photosynthetic and photoprotective pigment contents and sustained rates of photosynthesis and calcification during thermal stress (Béraud et al. 2013). Interestingly, in the latter study, nitrogen-enriched corals also showed a decreased organic carbon release potentially resulting from its higher utilization, together with the additional nitrogen, to form molecules of interest such as proteins (Béraud et al. 2013). Similarly, in our study, coral-associated diazotrophs may have benefited *A. hemprichii* by providing additional nitrogen, helping to maintain constant chlorophyll *a* levels and preventing dramatic decreases in photosynthesis. Further, nitrogen from N_2 fixation may have indirectly boosted molecule repair and photoprotective pigment synthesis (Béraud et al. 2013). The increase in N_2 fixation may not have sufficed the metabolic needs of *S. pistillata*, resulting in the onset of bleaching. On the contrary, N_2 fixation in this coral species may have resulted in a nutrient

imbalance and subsequent phosphate starvation of *Symbiodinium*, worsening the detrimental effects of high temperature.

In our coral nubbins, the steady increase in light N_2 fixation activity under increased temperature was concomitant with sustained photosynthesis, thus suggesting that nitrogenase (the oxygen-labile enzyme responsible for N_2 fixation; Berman-Frank et al. 2003) was protected from oxygen inhibition. Conversely, when the coral-algal symbiosis was impaired and bleaching occurred, we measured higher N_2 fixation in the dark, concomitant with oxygen consumption by the animal respiration. Therefore, the reversed pattern of daily N_2 fixation activity in recovering corals suggests a scenario in which different N_2 fixation strategies prevail in coral-associated diazotrophs confronted with different environmental and host health conditions. Future studies addressing the mechanisms by which these strategies prevail and the potential role of *Symbiodinium* in regulating the partnership of corals with diazotrophs will propel our understanding of the evolution of symbiotic nitrogen fixation and of coral holobiont ecology. Overall, our results importantly contribute to the understanding of the role that coral-associated microbes play in the holobiont under stressful conditions. Furthermore, our study highlights the paramount importance of the physiological and metabolic interconnections among the different partners of the coral-algal-prokaryote symbiosis in determining the fate of the holobiont facing climate change.

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Chapter IX

Ocean acidification rapidly reduces dinitrogen fixation associated with the hermatypic coral *Seriatopora hystrix*[*]

Abstract

Since productivity and growth of coral associated dinoflagellate algae is nitrogen (N)-limited, dinitrogen (N_2) fixation by coral-associated microbes is likely crucial for maintaining the coral-dinoflagellate symbiosis. It is thus essential to understand the effects future climate change will have on N_2 fixation by the coral holobiont. This laboratory study is the first to investigate short-term effects of ocean acidification on N_2 fixation activity associated with the tropical, hermatypic coral *Seriatopora hystrix* using the acetylene reduction assay in combination with calcification measurements. Findings reveal that simulated ocean acidification (pCO_2 1080 µatm) caused a rapid and significant decrease (53 %) in N_2 fixation rates associated with *S. hystrix* compared to the present day scenario (pCO_2 486 µatm). In addition, N_2 fixation associated with the coral holobiont showed a positive exponential relationship with its calcification rates. This suggests that even small declines in calcification rates of hermatypic corals under high CO_2 conditions may result in decreased N_2 fixation activity, since these two processes may compete for energy in the coral holobiont. Ultimately, an intensified N limitation in combination with a decline in skeletal growth may trigger a negative feedback loop on coral productivity exacerbating the negative long-term effects of ocean acidification.

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Introduction

Hermatypic corals are highly adapted to the oligotrophic waters in which they occur by forming a mutualistic symbiosis with dinoflagellate algae of the genus *Symbiodinium* (Muscatine & Porter 1977). Although this symbiosis enables an efficient internal recycling of nutrients, new nutrients (particularly bioavailable nitrogen) are needed to sustain net productivity and to compensate the loss of nutrients. New nitrogen (N) is acquired by the coral holobiont via capture of prey, assimilation of inorganic and organic N from the water column, and dinitrogen (N_2) fixation (Lesser *et al.* 2007, Grover *et al.* 2008). In this context, Lesser *et al.* (2004) for the first time detected endosymbiotic cyanobacteria in the coral *Montastraea cavernosa*. Recent research revealed that diazotrophs (N_2 fixing bacteria and archaea) are ubiquitous members of coral-associated microbial communities and form species-specific associations with their hosts (Lema *et al.* 2012, 2013, Olson & Lesser 2013). N_2 fixation activity has also been detected for several coral species, suggesting a high importance of this process in fulfilling the N demand of corals (reviewed in Fiore *et al.* 2010 and Cardini *et al.* 2014).

Since growth of *Symbiodinium* spp. is N limited, low DIN availability may be essential to maintain the stability of this symbiosis (Falkowski *et al.* 1993). On the other hand, *Symbiodinium* spp. is efficient in the uptake of fixed N (Kopp *et al.* 2013), and cell division rates are faster in corals that show high N_2 fixation activity (Lesser *et al.* 2007). Hence, N_2 fixation may play a key role in regulating the coral-dinoflagellate symbiosis. The effects of environmental changes, such as ocean acidification, on N_2 fixation associated with hermatypic corals have yet to be resolved. Several studies reported reduced calcification rates under high CO_2 conditions and reduced aragonite saturation (Cohen & Holcomb 2009, Ries *et al.* 2009, Crook *et al.* 2013). Even though positive as well as negative effects of ocean acidification on N_2 fixation activity by planktonic diazotrophs have been reported (Levitan *et al.* 2007, Czerny *et al.* 2009, Shi *et al.* 2012), there are no studies up to now investigating the effects of ocean acidification on N_2 fixation associated with hermatypic corals. Thus, in the present study we experimentally investigated the short-term response of N_2 fixation and calcification (light/dark) in the exemplary coral holobiont, *Seriatopora hystrix*, exposed to high CO_2 conditions as they may occur before 2100 according to the IPCC scenario RCP 8.5 (Riahi *et al.* 2007).

Methods

Model organism and sample preparation

The hermatypic coral *S. hystrix* was selected as model organism for this study as it is abundant, occurs in a wide range of habitats, and is frequently used in physiological studies (Sheppard 1987, Hoegh-Guldberg & Smith 1989, Bongaerts *et al.* 2011). The coral used for the experiment was acquired from the company De Jong Marinelife, Netherlands and was collected from shallow water depth of about 5 m in Indonesia. One individual colony was fragmented into 30 smaller colonies of an average size of 11.75 ± 1.12 cm^2 (mean ± SE) to remove genetic variability. All fragments were kept in a mesocosm holding tank (2000L) in the laboratory facilities of the Leibniz Centre for Tropical Marine Ecology (ZMT, Bremen) for two months prior to the measurements.

Experimental incubations

The seawater used for the CO_2 treatment was taken from the coral holding tank, filtered (0.1 µm, AcroPak™) and equilibrated with gas of defined CO_2 concentrations of either 486 ppmv (ambient) or 1080 ppmv (high). The resulting changes in seawater carbonate chemistry were calculated from pH (NBS) and total alkalinity (TA) using the CO_2 Sys Excel Macro (Lewis & Wallace 1998). pH (NBS) reading was obtained from a multiprobe (WTW 3430, Germany) and TA was measured by end-point titration with TW alpha plus (SI Analytic, Germany) using 0.5 M HCl. Corals were exposed to the CO_2 treatment in holding tanks for 20 h prior to the first incubations and for 24 h in between the first and the second incubation (salinity = 34‰, temperature = 26 ± 1 °C, PAR = 110 ± 5 quanta µmol s^{-1} m^{-2}). Additionally seawater at ambient or high CO_2 was used during the incubations, respective to the treatment. Calcification, photosynthesis, respiration and N_2 fixation rates were measured in two consecutive incubations. A total of 30 fragments were incubated with n = 15 for each CO_2 treatment level (ambient and high). Firstly, O_2 fluxes and calcification rates under treatment conditions (seawater of ambient or high CO_2) were quantified during the same incubation. Oxygen fluxes of the coral fragments were measured during light (PAR = 110 ± 5 quanta µmol s^{-1} m^{-2}) and dark incubations (less than 2 h each to avoid supersaturation of O_2) in 250 mL glass chambers by constant logging of O_2 concentrations using O_2 optodes (Firesting, PyroScience Sensor Technology, Germany). Water samples of 50 ml were collected from each chamber before and after each incubation (light/dark) to measure calcification rates. All coral

fragments were returned to the treatment aquaria of high or ambient CO_2, according to the treatment, for 24 h before start of the second incubation.

In the second incubation, the acetylene reduction technique was used to quantify N_2 fixation rates of the coral fragments (Hardy *et al.* 1968, Wilson *et al.* 2012). Coral fragments were incubated in 1 L glass chambers filled with 800 mL of treatment water (ambient or high CO_2 respectively), of which 10 % (80 mL) were previously saturated with acetylene (C_2H_2) to improve equilibration in the chamber. Also, 10 % (20 mL) of the 200 ml headspace were replaced with C_2H_2 gas after the chambers were sealed gastight. The incubation lasted for 22 h, starting with a 12 h dark phase followed by a 10 h light phase. During incubation, chambers were kept at constant temperature of 26.0 ± 0.3 °C. Gas samples of 1 mL were taken from the headspace after time intervals of 0, 4, 12 and 22 h, and collected in 2 mL glass vials previously filled with deionized water. Vials were stored frozen upside down until analysis to prevent any leakage from the septa.

Sample Analyses

Respiration and gross photosynthesis rates were calculated from the incubation periods which showed linear changes in O_2 concentration. Changes in the total alkalinity of the water samples before and after the incubations were converted into calcification rates using the alkalinity anomaly technique (Chrisholm & Gattuso 1991). Nitrogen fixation rates were calculated as ethylene (C_2H_4) evolution rates and not converted into actual fixation rates of N_2, as we acknowledge that there is an ongoing discussion about the correct conversion factor in the scientific community (Nohrstedt 1983, Wilson *et al.* 2012). C_2H_4 concentrations in the gas samples were quantified by gas chromatography (Varian 3800 with AL203/KCL 50x0.53 column and FID detector). Changes in C_2H_4 concentration were converted into C_2H_4 evolution rates according to Breitbarth *et al.* (2004). N_2 fixation rates showed a distinct initial lag phase during the first 4 h of incubation. This is a common phenomenon during acetylene reduction assays (Zuberer & Silver 1978, Gallon & Hamadi 1984, Shashar *et al.* 1994). Hence, N_2 fixation rates for the dark were calculated based on C_2H_4 concentration differences during the second time interval (4 to 12 h) without considering the first 4 h of incubation. Light N_2 fixation rates were calculated based on concentration differences between 12 and 22 h of incubation time.

Photosynthesis, respiration, calcification and N_2 fixation rates were corrected for seawater control (n = 6) signals and normalized to incubation time and coral surface area, which was quantified by advanced geometry (Naumann *et al.* 2009).

Data Analysis

All statistical analyses were conducted with R version 3.0.2. Differences in N_2 fixation rates were analysed using generalized mixed effect linear models (GLMM) with gamma distribution and an inverse link function taking into account minor fluctuations in water temperatures during the incubations to increase the fit of the model. O_2 fluxes, calcification rates and the relationship of calcification with N_2 fixation rates were also analysed with generalized linear models (GLM) with gamma distribution and an inverse link function. To meet the assumptions of gamma distribution, O_2 fluxes, calcification and N_2 fixation rates were (x+1) transformed. All data were corrected for outliers using the Dixon test.

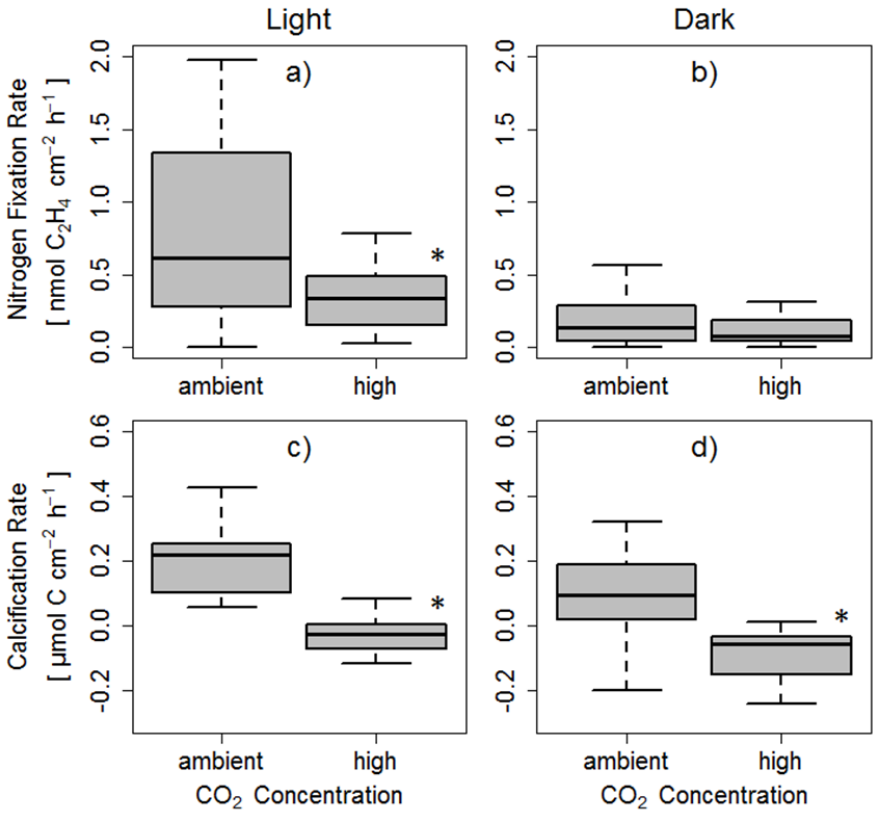

Figure 9.1. Boxplots for N_2 fixation rates (a,b) and calcification rates (c,d) of S. hystrix depending on the CO_2 treatment (n = 15 for N_2 fixation rates and n = 13 for calcification rates) for the light (a,c) period and dark (b,d) period. All N_2 fixation rates are expressed as ethylene (C_2H_4) production rates. All rates were corrected for seawater control and normalized to incubation time and coral surface area. Boxes show upper and lower quartile as well as median of data distribution. Whiskers indicate data points within 1.5 times the interquartile range from the box. Boxplots marked by asterisks (*) are significantly different from each other.

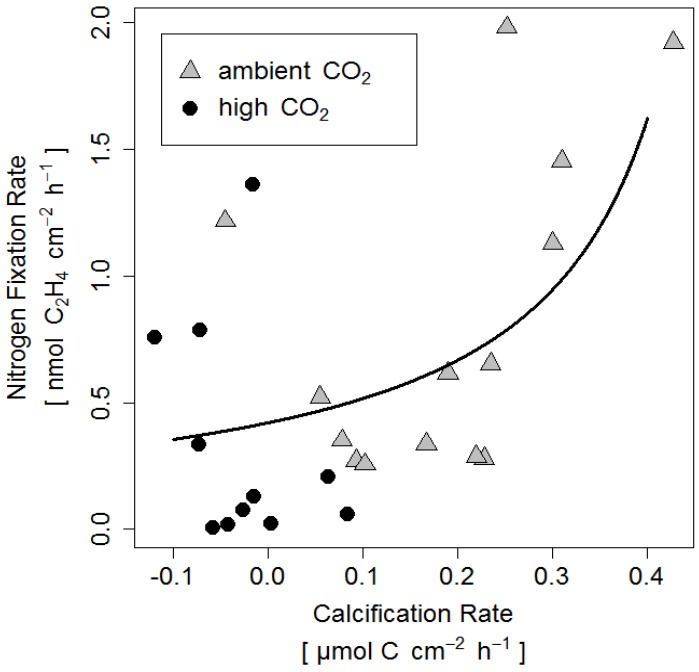

Figure 9.2. Relationship of N_2 fixation rates and calcification rates of S. hystrix incubated in the light under high CO_2 (black points) and control conditions (grey triangles). All rates were corrected for seawater controls and normalized to incubation time and coral surface area. Black curve indicates the best-fitting model ($\chi^2_{(1, N=25)}$ = 5.21, p = 0.03, Mc Fadden's R^2 = 0.862).

Results

The seawater carbonate system following the manipulation of CO_2 concentrations showed significant differences. At a total alkalinity of 1784 ± 36 µmol kg seawater^{-1} ambient CO_2 concentrations resulted in an aragonite saturation state (Ω Ar) of 1.9 ± 0.1 at a pH of 8.02, whereas high CO_2 concentrations showed an Ω Ar of 1.0 ± 0 at a pH of 7.71.

Short term exposure to high CO_2 concentrations revealed strong effects on the physiology of fragments of *S. hystrix* compared to the fragments incubated under ambient CO_2 concentrations. N_2 fixation activity (acetylene reduction) was variable, but measurable in all coral fragments. Rates were higher (3-4 times) in the light than in the dark independently of the treatment applied ($\chi^2_{(1, N=30)}$ = 22.839, p << 0.001). N_2 fixation rates ranged from 0.04 to 1.98 nmol C_2H_4 cm^{-2} h^{-1} during the light period and from 0.00 to 0.56 nmol C_2H_4 cm^{-2} h^{-1} during the dark period (Fig 1a,b). High

CO_2 levels caused a significant decline (53%) in the N_2 fixation rates of the coral holobiont in the light ($\chi^2_{(1, N=30)} = 6.8271, p<0.01$), but not in the dark, because rates were too low to indicate any significant differences ($\chi^2_{(1, N=30)} = 0.8311$, $p=0.36$). N_2 fixation rates of the coral nubbins in the light were 0.83 ± 0.16 nmol C_2H_4 cm^{-2} h^{-1} (means ± SE) under ambient CO_2 concentrations compared to 0.39 ± 0.09 nmol C_2H_4 cm^{-2} h^{-1} under high CO_2 concentrations.

Overall, calcification rates ranged from -0.12 to 0.42 μmol C cm^{-2} h^{-1} in the light and -0.24 to 0.32 μmol C cm^{-2} h^{-1} in the dark period (Fig. 1c,d). Calcification rates showed a pronounced response to differences in CO_2 concentrations. Calcification was significantly reduced under high CO_2 conditions compared to ambient CO_2 levels both in the light ($\chi^2_{(1, N=26)} = 26.651$, $p << 0.001$) and in the dark period ($\chi^2_{(1, N=26)} = 4.55$, $p << 0.001$). At ambient CO_2 concentrations, calcification rates were 0.20 ± 0.03 μmol C cm^{-2} h^{-1} in the light and 0.09 ± 0.04 μmol C cm^{-2} h^{-1}in the dark period. At high CO_2 concentrations, calcification rates were -0.01 ± 0.03 μmol C cm^{-2} h^{-1}in the light and -0.08 ± 0.02 μmol C cm^{-2} h^{-1}in the dark. Since both calcification and N_2 fixation decreased under the ocean acidification scenario, the relationship between these two processes was investigated (Fig. 2). This revealed a positive exponential correlation of N_2 fixation activity and calcification rates in coral fragments incubated in the light ($\chi^2_{(1, N=25)} = 5.21$, $p = 0.02$) as opposed to dark incubations, where the relationship was not significant ($\chi^2_{(1, N=25)} = 0.35$, $p = 0.55$).

Differences in CO_2 concentrations had no significant effect on gross photosynthesis ($\chi^2_{(1, N=30)} = 0.01$, $p = 0.90$) and respiration rates ($\chi^2_{(1, N=30)} = 0.18$, $p = 0.67$) of the coral nubbins. Mean gross photosynthesis was 0.50 ± 0.04 μmol O_2 cm^{-2} h^{-1} under high CO_2 compared to 0.49 ± 0.05 μmol O_2 cm^{-2} h^{-1} under ambient CO_2 conditions. Respirations rates were -0.30 ± 0.03 μmol O_2 cm^{-2} h^{-1} at high CO_2 and -0.28 ± 0.2 μmol O_2 cm^{-2} h^{-1} at low CO_2 conditions respectively.

Discussion

This is the first study showing N_2 fixation associated with *S. hystrix* and demonstrating the effect of elevated CO_2 levels on N_2 fixation. N_2 fixation has been described for several other coral species, with a pronounced variation between and within species (Williams *et al.* 1987, Shashar *et al.* 1994, Lesser *et al.* 2007). To control for the intra-specific differences, manipulative experiments need to use individuals of identical genotype (Mascarelli & Bunkley-Williams 1999). All experiments in this study where conducted with coral colonies from the same colony. Thus the observed physiological changes can be referred back to treatment conditions.

N_2 fixation is an energy-intensive process (McNarry & Burris 1962). Shashar *et al.* (1994) found that N_2 fixation activity was inhibited in corals when photosynthesis was blocked with DCMU, but could be restored when glucose was added to the incubation water. This suggests that coral associated N_2 fixation strongly depends on photosynthetically fixed carbon to fulfil its energetic demands. In the present study, N_2 fixation rates were three to four times higher during the light compared to the dark. This is likely explained by increased availability of fixed carbon by photosynthesis during the light phase. N_2 fixation occurred during times of net O_2 evolution, although O_2 is known to inhibit this process (Gallon 1981). There are different mechanisms by which N_2 fixation can take place at times of O_2 evolution (Gallon 1981). In coral reef sponges for example symbiotic non-heterocystous cyanobacteria, which depend on O_2 for their N_2 fixation, have been suggested to explain high N_2 fixation activity under aerobic conditions (Wilkinson & Fay 1979, Mohamed *et al.* 2008).

N_2 fixation rates were significantly reduced in the ocean acidification treatment compared to the ambient scenario in the light. Other studies reported an increase of N_2 fixation activity under elevated CO_2 conditions for planktonic cyanobacteria due to increased photosynthetic carbon fixation by overcoming of CO_2 limitation (Hutchins *et al.* 2007, Garcia *et al.* 2013). This may be the case for planktonic autotrophic diazotrophs, but CO_2 limitation is unlikely to occur in the *S. hystrix* holobiont due to respiration by the coral host. Reduced N_2 fixation rates under elevated CO_2 concentrations have only been described in the planktonic cyanobacterium *Trichodesmium* in combination with low iron availability (Shi *et al.* 2012). Since the experiments carried out in the present study took place in laboratory conditions, it is unlikely that iron limitation caused the lowering of fixation rates in the short time span of the experiment described in the present study. Hence, there has to be another cause for the effects observed. Along with N_2 fixation, calcification of *S. hystrix* was significantly reduced during both light and dark periods. The significant positive correlation between both processes during the light may suggest an indirect linkage of the two processes in the holobiont.

The reduced calcification rates are in good agreement with previous studies reporting similar effects under low pH conditions due to lowered aragonite saturation state (Orr *et al.* 2005, Anthony *et al.* 2008, Kleypas & Yates 2009). Since N_2 fixation and calcification are energy-intensive mechanisms, they likely compete for energy within the coral holobiont. The lowering in the aragonite saturation state makes the calcification process more energy consuming (Marubini *et al.* 2001, Hohn & Merico 2012). Since gross and net photosynthesis were not significantly different between treatments, the increased energy demand by calcification at high CO_2 conditions may

create an energy deficit in the coral holobiont. Subsequently, this may also reduce the energy available for heterotrophic diazotrophs in the coral tissue, thereby explaining the decrease in N_2 fixation activity at high CO_2 conditions. Although Anthony *et al.* (2008) reported a loss of coral productivity at lower seawater pH during long term experiments, there was no effect of elevated CO_2 on photosynthesis and respiration of the fragments used in the present study, probably due to the short time span of the incubations. It is hence likely that the described long term drop in productivity will amplify the effects of ocean acidification on N_2 fixation and calcification even more. This is the first evidence that coral associated N_2 fixation can be affected by ocean acidification. The observed decline in N_2 fixation may result in N starvation for both the coral and *Symbiodinium* spp. Together with a reduced skeletal growth this suggests a negative feedback loop for the productivity of the holobiont. The reduction in N_2 fixation may thus exacerbate negative long-term effects of ocean acidification for coral reef functioning. Finally, these findings highlight the importance of N_2 fixation as key process for understanding the response of the coral holobiont to environmental stressors such as ocean acidification. To improve the understanding of interactions between diazotrophs, *Symbiodinium* spp. and the coral host an interdisciplinary approach is needed, combining ecological and microbiological aspects.

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Chapter X

Thesis Conclusion

Dinitrogen fixation by coral reef primary producers

Coral reefs are environments of high N_2 fixation and it has been suggested that this additional source of new bioavailable N may support primary production and coral reef functioning in oligotrophic tropical waters. Most studies have focused on the investigation of N_2 fixation activity by a single coral reef benthic group (e.g. reef sands, macroalgae, hard corals, microbial mats) making it difficult to compare the different benthic groups in their relative importance and contribution to total N_2 fixation within a reef. Therefore, the first goal of this thesis was to conduct a detailed survey concerning N_2 fixation activity associated with the most dominant benthic groups in a fringing reef in the Northern Gulf of Aqaba. A total of 14 different organisms and substrates were investigated; belonging to 10 benthic groups that occupy > 90 % of the reef benthos (Table 10.1). This data was presented in chapters II, III, IV and V, and have been brought together for chapter X to compare and contrast the N_2 fixation activities of the individual benthic groups. Additionally, the N_2 fixation rates averaged for each benthic group were multiplied by the relative abundance of each benthic group on the reef and by a benthic group-specific 2D:3D conversion factor (Naumann et al. 2012, van Hoytema et al. unpublished data) in order to estimate N_2 fixation of the total reef benthos and calculate the respective contribution of each benthic group to total benthic N_2 fixation.

Firstly, this thesis shows that N_2 fixation is a ubiquitous process on a coral reef in the Northern Gulf of Aqaba. All investigated benthic groups revealed N_2 fixation activity, although, actual rates depended on the benthic group and environment being examined. Highest N_2 fixation rates per unit surface area were associated with microbial mats, turf algae, coral rock and reef sands. Collectively their total contribution to benthic N_2 fixation ranged from 92 % at 5 m to 65 % at 20 m (Table 10.2). This agrees with previous studies that have highlighted these groups as important contributors to the input of new N to the reef ecosystem (Larkum et al. 1988, Shashar et al. 1994,

Williams & Carpenter 1998, Charpy-Roubaud et al. 2001, Davey et al. 2008, Charpy et al. 2010). Of particular importance are sand-dominated areas (i.e. lagoons) which can contribute up to 70 % of reef N_2 fixation (Shashar et al. 1994). In my study area, at 5 m water depth, carbonate sand was the main benthic component (51 % benthic cover) and contributed 43 % to total benthic N_2 fixation. At other reef areas (10 and 20 m water depth) the carbonate sand cover ranged from 3-16 % corresponding with a much lower contribution to total N_2 fixation (i.e. 3-19 %).

Table 10.1 Annual average (mean ± SE) of N_2 fixation rates (NF in μmol N_2 m^{-2} h^{-1} using a C_2H_4:N_2 conversion factors of 4) and benthic cover (%) at 1, 5, 10 and 20 m water depth by the different reef-framework and reef sand associated benthic groups in a fringing reef in the Gulf of Aqaba.

Benthic groups	Thesis chapter	NF	Benthic cover (%)		
			5 m	10 m	20 m
Reef-framework associated					
Hard corals *1	4	0.39 ± 0.05	16.7	36.5	63.7
Soft corals *2	5	0.09 ± 0.02	3.0	21.6	6.6
Sponges *3	3	0.45 ± 0.11	1.5	1.4	1.3
Macroalgae *4	u.d.	2.23 ± 0.46	3.5	3.4	3.6
Turf algae	3	10.91 ± 1.79	6.5	4.4	4.9
Coral rock	3	8.71 ± 1.32	7.0	8.5	8.1
Reef sand associated					
Carbonate sands	2	7.20 ± 1.02	50.7	16.2	2.6
Silicate sands	2	3.80 ± 0.37	n.p.	n.p.	n.p.
Macroalgae *5	u.d.	2.68 ± 0.60	0.0	1.2	0.2
Microbial mats	2	29.86 ± 2.90	1.1	0.2	0.2

n.p.) not part of the transect; *1) *Acropora* sp., *Goniastraea* sp., *Pocillopora* sp., *Stylophora* sp.;
*2) *Sarcophyton* sp., Xeniidae; *3) *Mycale fistulifera*; *4) *Lobophora* sp.; *5) *Caulerpa* sp.;
u.d.) unpublished data and not included in the thesis chapters

Interestingly, I found that in sand, N_2 fixation is also determined by the predominant sand type. At the study site two different sand types (carbonate and silicate sands) co-occur, and previous studies have described for these two sands specific microbial communities (abundance, activity) and specific physico-chemical characteristics (i.e. grain size, porosity, permeability and transparency to light) that all influence benthic-pelagic coupling, the content and degradation of organic matter in sands, and thus nutrient recycling processes within the reef (Rasheed et al. 2003a, 2003b, Schöttner

et al. 2011, Huettel et al. 2014). Similarly, N_2 fixation activities may differ between the two sand types, and the findings of this thesis revealed significantly higher rates associated with carbonate than with silicate sands (Chapter II). The higher organic carbon content reported for carbonate sands compared to silicate sands, likely facilitates the high energetic requirements for N_2 fixation. Conversely, the high organic N content in carbonate sands may result from its high N_2 fixation activity. Finally, this study suggests that N_2 fixation in reef sands, particularly carbonate sands, may importantly fuel the N requirement of the total reef benthos by sustaining the release of fixed N compounds to the overlaying water. Since N_2 fixation rates were found to be significantly lower in silicate sands, not only the total sand area on the reef but also the predominant sand type determines the input of new N to the reef ecosystem.

Table 10.2 The % contribution of the different reef-framework and reef sand associated benthic groups to total benthic N_2 fixation is calculated by multiplying the N_2 fixation rate by the relative substratum abundance and by specific 2D:3D conversion factors.

Benthi groups	**2D:3D**	**Contribution (%) to total benthic N_2 fixation**		
		5 m	**10 m**	**20 m**
Reef-framework associated				
Hard corals *1	6.6	5.1	15.5	29.4
Soft corals *2	15.5	0.5	5.1	1.7
Sponges *3	3.1	0.3	0.3	0.3
Macroalgae *4	2.5	2.3	3.1	3.6
Turf algae	3.1	26.2	24.5	29.6
Coral rock	2.5	18.2	30.4	31.5
Reef sand associated				
Carbonate sands	1.0	43.5	19.1	3.3
Macroalgae *5	1.8	0.0	0.9	0.1
Microbial mats	1.0	3.9	1.0	0.5

*1) *Acropora* sp., *Goniastraea* sp., *Pocillopora* sp., *Stylophora* sp.; *2) *Sarcophyton* sp., Xeniidae;
*3) *Mycale fistulifera*; *4) *Lobophora* sp.; *5) *Caulerpa* sp.

N_2 fixation per unit surface area of hard corals, soft corals and sponges were in the lower range among all investigated benthic groups. Nevertheless, the contribution of hard corals to total benthic N_2 fixation is in the same range as turf algae, coral rock and reef sands when taking the three-dimensional surface structure and high benthic cover of hard corals into account (Table 10.1 and 10.2). The N fixed by hard coral-associated diazotrophs has been suggested to facilitate the

photometabolic N demand of the endosymbiotic zooxanthellae in oligotrophic waters with a positive feedback for the coral host metabolism. Besides benefiting the coral-zooxanthellae symbiosis, a recent study showed that coral-derived organic matter released to the seawater contains high amounts of fixed N derived from N_2 fixation (Grover et al. 2014). Hard corals release up to 40 % of the photosynthetically fixed carbon as particulate or dissolved organic matter into surrounding reef waters (Crossland et al. 1980). This coral-derived organic matter fulfils important functions for the coral organism (i.e. protection against desiccation, sedimentation, pathogens), and for the coral reef ecosystem by initiating recycling processes that retain energy and essential nutrients within the reef (Krupp 1984, Riegl & Branch 1995, Ritchie 2006, Naumann et al. 2009, Wild et al. 2004). Thus, the high release of organic matter and the high contribution of N_2 fixation associated with hard corals to total benthic N_2 fixation underlines the significant role of hard corals for oligotrophic reef ecosystems and suggests that N_2 fixation may also influence their role as allogenic ecosystem engineer. While N_2 fixation and organic matter release have previously been studied for several hard coral species, this thesis investigated both processes on soft corals, an abundant, but understudied, benthic group in many coral reefs worldwide. The results show that soft corals had substantially lower N_2 fixation and organic matter release rates in comparison to hard corals (Chapter V and VI). Potential reasons for the lower N_2 fixation activity may be the lack of endolithic diazotrophs, and thus, a generally lower abundance of diazotrophs compared to hard corals. Reduced N availability from N_2 fixation may subsequently affect organic matter fluxes, particularly the release of organic N, although a direct link between N_2 fixation and organic matter fluxes associated with soft corals still needs to be investigated. Instead, this thesis tested the effect of elevated ammonium and phosphate availability on soft coral-derived organic matter fluxes. While the addition of ammonium had no effect on organic matter fluxes, phosphate addition stimulated particulate organic N release by the soft corals. Since primary productivity and chlorophyll *a* content of the zooxanthellae also increased in response to phosphate addition, it clearly indicates that there is a phosphate limitation on the zooxanthellae and suggests that the potential associated diazotrophs were also phosphate limited. Therefore, the increased particulate organic N release may have been caused by stimulated N_2 fixation activities under phosphate enriched conditions. In the study area at 10 m water depth, soft corals represent the secondmost abundant benthic group after hard corals. Despite covering 22 % of the reef benthos soft corals contributed only 5 % to total benthic N_2 fixation. Thus, input of new N via N_2 fixation, as well as ecosystem engineering *via* organic matter release, may be greatly reduced in soft coral dominated reefs compared to those dominated by hard corals.

Lastly, the benthic abundance and N_2 fixation activity of each benthic group was used to estimate the total benthic N_2 fixation in 5, 10 and 20 m water depth on the reef. Total benthic N_2 fixation reached 8.4, 6.1 and 5.6 μmol N_2 m^{-2} reef h^{-1} on annual average at 5, 10 and 20 m, respectively. To my knowledge only two previous studies have calculated N_2 fixation for a total reef community (Larkum et al. 1988, Shashar et al.1994). Larkum et al. (1988) estimated comparable rates of 3.3 to 6.5 μmol N_2 m^{-2} reef h^{-1} for One Tree Reef (southern Great Barrier Reef), while Shashar et al. (1994) found rates of 24 to 41 μmol N_2 m^{-2} reef h^{-1}, up to 5-fold higher rates for a reef in the Gulf of Aqaba (Eilat, Israel). Shashar et al. (1994) also reported 1.7 higher rates in the sandy reef lagoon compared to the reef flat or the fore reef area. This areal pattern of N_2 fixation is confirmed by the present study as up to 1.5 fold higher N_2 fixation rates per square meter reef were calculated for the sand dominated area at 5 m water depth compared to the hard coral dominated areas in 10 and 20 m water depth. Thus, depending on the size, sand dominated reef areas (i.e. reef lagoons) may indeed account for the major N input *via* N_2 fixation within the entire reef.

Dinitrogen fixation under changing environmental conditions

The second theme of this thesis focused on the effect of changing environmental conditions on N_2 fixation associated with the different benthic groups. Seasonal variations in environmental conditions (Chapter II to V) and the effect of single key environmental factors (Chapter VII, VIII and IX) were tested. In order to compare the seasonal responses of the benthic groups, for each group the seasonal mean N_2 fixation rates was divided by the annual mean N_2 fixation rate (Table 10.3). Generally, the seasonal response of N_2 fixation was different for the reef-framework compared to the reef sand associated benthic groups. All reef-framework associated benthic groups revealed 2 to 3 fold higher N_2 fixation rates during summer compared to the annual average, while the N_2 fixation rates during winter, spring and autumn were lower than the annual average. In contrast, the reef sand associated benthic groups revealed up to 2 fold higher N_2 fixation rates during both spring and summer. This suggests that different environmental factors are controlling N_2 fixation activities in reef-framework and reef sand associated diazotrophic communities.

Table 10.3 Ratio between seasonal (winter, spring, summer, autumn) and annual N_2 fixation rates (given in Table 10.1) associated with the different reef-framework and reef sand associated benthic groups in a fringing reef of the Gulf of Aqaba. The ratio for each benthic group and each season is presented. For actual seasonal N_2 fixation rates see Figure 3.1 (hard corals), Figure 4.2 (soft corals), Figure 3.1 (Sponges, turf algae and coral rock) and Figure 2.2 (carbonate sands, silicate sands and microbial mats).

Benthic groups	**Dinitrogen fixation** (seasonal mean:annual mean)			
	Winter	Spring	Summer	Autumn
Reef-framework associated				
Hard corals *1	0.31	0.48	2.49	0.72
Soft corals *2	0.48	0.74	2.63	0.38
Sponges *3	0.13	0.44	3.08	0.69
Macroalgae *4	0.20	0.64	2.51	0.64
Turf algae	0.41	0.61	2.56	0.62
Coral rock	0.13	0.89	1.99	0.76
Reef sand associated				
Carbonate sands	0.41	1.82	1.50	0.40
Silicate sands	1.59	0.53	1.37	0.63
Macroalgae *5	0.01	1.88	1.51	0.66
Microbial mats	0.44	1.71	0.74	0.96

*1) *Acropora* sp., *Goniastraea* sp., *Pocillopora* sp., *Stylophora* sp.; *2) *Sarcophyton* sp., Xeniidae; *3) *Mycale fistulifera*; *4) *Lobophora* sp.; *5) *Caulerpa* sp.

The spring season in the Gulf of Aqaba is characterized by low water temperatures, high nutrient concentrations (deep-water mixing) and high light availabilities, while summer displays high water temperature, low nutrient concentrations (thermal stratification), and only slightly higher light availabilities compared to spring (for details see Table 2.2). The differences in water temperature and nutrient concentrations but similarity in ambient light availabilities between the spring and summer seasons suggest that light is likely the main environmental factor controlling reef sand associated N_2 fixation. Light availability indirectly controls N_2 fixation by stimulating photosynthesis the energy-generating process in phototrophic diazotrophs (Carpenter & Capone 2008). In order to examine the individual effect of light on reef sand associated N_2 fixation, carbonate sand was collected along a depth-mediated light gradient and incubated for N_2 fixation activity under depth-adjusted light intensities. Indeed, results show that reef sand-associated N_2 fixation activity was 3 fold higher under light intensities of 400 compared to 110 µmol quanta m^{-2} s^{-1} (Bednarz et al. unpublished data). A positive relationship between light availability and carbonate sand associated N_2 fixation activity has been previously demonstrated (Charpy-Roubaud et al. 2001,

Charpy et al. 2007, Werner et al. 2008) and strongly indicates the dominance of phototrophic diazotrophs in reef sand associated communities. In contrast, the strong increase in N_2 fixation by reef-framework associated benthic groups only during summer indicates that the increased water temperature (+5°C compared to spring) and reduced nutrient concentrations were the main controlling environmental factors. Elevated temperature stimulates enzyme-mediated reactions such as N_2 fixation (Capone et al. 1997, 2005), while dissolved inorganic nitrogen (DIN) concentrations above a certain threshold (~1 μM) makes the energy-demanding N_2 fixation process energetically inefficient (Carpenter & Capone 2008). DIN concentrations of coral reef waters in the study area were 2 to 5 times lower during summer (0.20 μM) compared to the other three seasons (0.43 to 1.03 μM). The manipulation of single environmental factors in two experiments on hard corals confirmed, that increased water temperature (+6°C) lead to a drastic increase in coral-associated N_2 fixation rates (Chapter VIII), while no change in coral-associated N_2 fixation was observed under light intensities ranging from 110 to 680 μmol quanta m^{-2} s^{-1} (Chapter VII).

Overall, the findings from this thesis highlight that the seasonal response of N_2 fixation is highly dependent in the benthic group likely due to different diazotrophic communities associated with the reef-framework or the reef sands (Charpy-Roubaud et al. 2001). Particularly during the nutrient-depleted summer season benthic N_2 fixation may play a key role for sustaining primary production in coral reefs. We calculated that fixed N from N_2 fixation contributes between 11 and 17 % to the photometabolic N demand of different benthic groups (hard corals, turf algae, coral rock and reef sand) during summer, while only 1 to 7 % was provided during the other seasons. The low N availability and the low N_2 fixation activity in the water column of the Gulf of Aqaba (Foster et al. 2009, Rahav et al. 2013), as well as the importance of N for cell maintenance, growth and reproduction, further highlight the importance of benthic N_2 fixation for coral reef functioning in oligotrophic areas.

Outlook

Coral reefs are well adapted to seasonally changing environmental conditions. However, these ecosystems are increasingly impacted by global (i.e. ocean warming and ocean acidification) and local (i.e. eutrophication) stressors (Hough-Guldberg et al. 2007, Burke et al. 2011) which is leading to coral reef degradation worldwide. Recent studies have demonstrated shifts in the diversity of coral-associated microbial assemblages, including diazotrophs, in response to elevated water temperature and pCO_2 conditions (Morrow et al. 2014, Santos et al. 2014). These results

suggest concomitant effects on important microbial-mediated processes, such as N_2 fixation. The present thesis shows for the first time that thermal stress resulted in increased coral-associated N_2 fixation rates, while increased pCO_2 as predicted by future ocean acidification scenarios had an opposite effect. Therefore, the question arises how the combination of both stressors will interact to affect N_2 fixation and the provision of important nutrients to the coral host during stressful conditions. Furthermore, previous studies have demonstrated severe impacts of eutrophication on diazotrophs from the open ocean (reviewed in Sohm et al. 2011). As coastal environments are typically more affected by eutrophication than pelagic environments, the impact of eutrophication on N_2 fixation in corals reefs needs to be investigated. Global and local stressors can affect diazotrophs in coral reefs either by directly impacting their N_2 fixation activity or by indirectly altering their diversity because of shifts in the community of their benthic eukaryotic hosts, eventually resulting in changes in overall reef N_2 fixation. While shifts from hard coral to algae dominated reefs are the most frequently reported community changes, alternative changes to reefs dominated by sponges or soft corals can also occur (Tilot et al. 2008, Norström et al. 2009). This thesis provides evidence that benthic N_2 fixation and organic matter release rates may be greatly reduced after phase-shifts from hard to soft coral dominance. Since N_2 fixation and coral-derived organic matter release are key processes for the functioning and productivity of oligotrophic reef ecosystems it is important to understand how these will be affected when reefs are under conditions of alternative ecological states. Furthermore, studies examining the combined effects of local and global stressor on these processes are the key to understand how reef biogeochemical cycles will adjust to future climate change. Finally, a multidisciplinary approach combining physiological, ecological and microbiological methods is required if we want to understand the multifarious changes that reefs are experiencing from the microbial to the ecosystem level.

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Abstracts of additional manuscripts

(These additional manuscripts are related to this thesis but are not included into it.)

1)

Marine Eutrophication – Overview of Indicators

Jessen C, **Bednarz VN**, Rix L, Teichberg M, Wild C

Accepted for publication as book chapter in the Springer book "Environmental Indicators" (ed. Robert Armon), 2014

Eutrophication is one of the key local stressors for coastal marine ecosystems, particularly in those locations with many estuaries, intense coastal development or agriculture, and a lack of coastal forests or mangroves. The land-derived import of not only inorganic nutrients, such as nitrate and phosphate, but also particulate and dissolved organic matter (POM and DOM) affects the physiology and growth of marine organisms with ensuing effects on pelagic and benthic community structures, as well as cascading effects on ecosystem functioning. Indicators for marine eutrophication are therefore not only key water quality parameters (inorganic and organic nutrient concentrations, oxygen and chlorophyll availability, and biological oxygen demand), but also benthic status and process parameters, such as relative cover and growth rates of indicator algae, invertebrate recruitment, sedimentary oxygen demand, and interactions between indicator organisms. The primary future challenge lies in understanding the interaction between marine eutrophication and the two main marine consequences of climate change, ocean warming, and acidification. Management action should focus on increasing the efficiency of nutrient usage in industry and agriculture, while at the same time minimizing the input of nutrients into marine ecosystems in order to mitigate the negative effects of eutrophication on the marine realm.

2)

The influence of seasonality on benthic carbon fixation in a Northern Red Sea coral reef

van Hoytema N, **Bednarz VN**, Cardini U, Naumann MS, Al-Horani F, Wild C

Under review at Marine Ecology Progress Series

Coral reefs in the Northern Red Sea experience seasonal variations in environmental factors such as water temperature, light and nutrient availability. This offers the opportunity to study the effects of environmental variation on coral reef primary production. The present study therefore quantified, during all four seasons, gross photosynthesis (Pg) of the dominant benthic primary producers (corals, macroalgae, turf algae, sedimentary microphytobenthos, coral rock, and cyanobacterial mats) from a Jordanian fringing reef by measuring net photosynthesis and respiration using stirred respirometry chambers. Findings revealed that annual mean Pg was highest for the macroalgae *Caulerpa* sp. and the hard coral *Goniastrea* sp. (752 and 679 nmol C cm^{-2} h^{-1} respectively). Least productive were the sedimentary microphytobenthos and macroalgae Lobophora (241 and 269 nmol C cm^{-2} h^{-1} respectively). Sedimentary microphytobenthos exhibited the strongest response to seasonality: 3.5 times higher Pg in spring than winter. Pg of primary producers that responded significantly to seasonality increased with increasing light availability from winter to spring. Primary producers were either able to maintain their increased Pg from spring into summer (hard corals *Stylophora* sp. and *Goniastrea* sp., and soft coral *Sarcophyton* sp.), or their Pg was not significantly increased in summer compared to winter (soft coral Xeniidae, sedimentary microphytobenthos). The former could potentially alleviate nutrient limitation in oligotrophic summer (via e.g. heterotrophic feeding), while the latter could not. Relatively high Pg and benthic cover make hard corals most important for reef primary production. Potential phase shifts from hard corals to turf algae or soft corals, would thus likely decrease local reef productivity.

3)

Seasonal variability of carbon and dinitrogen fixation in the water column above a Red Sea coral reef (Gulf of Aqaba)

van Hoytema N, Cardini U, **Bednarz VN**, Rix L, Naumann MS, Al-Horani F, Wild C

In preparation for Limnology and Oceanography

The Northern Red Sea, because of is high-latitude location, experiences strong seasonality with likely pronounced changes in both key status (temperature, inorganic and organic nutrient concentrations) and process parameters (carbon (C) and dinitrogen (N_2) fixation) of the water column. However, knowledge of interrelations between these parameters is lacking. This study thus measured the above mentioned parameters in high temporal resolution in a comparative approach between mixed (January – April) and stratified (September – November) water column scenarios in 2013. Findings revealed that inorganic nutrient concentrations were significantly lower in the stratified than in the mixed season. Concurrently, planktonic C fixation decreased significantly 4-fold from mixed to stratified season, while planktonic N_2 fixation did not change significantly. C fixation over the year was most strongly and positively correlated to inorganic nutrient concentrations, while N_2 fixation was positively correlated to C fixation in the mixed season and dissolved organic carbon concentration in the stratified season, suggesting a community shift from autotrophic to heterotrophic nitrogen fixing microbes between seasons. N_2 fixation provided 3.4 % of N needed for C fixation in the mixed season, but this contribution increased drastically to 20.8 % in the stratified season, indicating that planktonic N_2 fixation is an important source of essential N to the autotrophic plankton community during oligotrophic conditions.

4)

Seasonal variation of carbon fluxes induced by dominant hard and soft corals in a northern Red Sea coral reef

van Hoytema N, **Bednarz VN**, Cardini U, Rix L, Naumann MS, Al-Horani F, Wild C

In preparation for Coral Reefs

Corals play a vital role in the fixation and cycling of carbon (C) in coral reefs. However, studies on C cycling that include both hard and soft corals, and the natural environmental change they experience, are scarce. Therefore, this comprehensive study quantified C fluxes induced by the dominant hard and soft corals of a high latitude fringing reef in the northern Red Sea while they were exposed to typical pronounced seasonal variability in key environmental factors. Physiological measurements of photosynthesis (PS), respiration (R), calcification (G), and particulate and dissolved organic carbon (POC and DOC, respectively) fluxes were performed in all four seasons. Measurements were supplemented by in situ monitoring of key environmental factors such as temperature, light availability, and inorganic nutrient concentrations. Findings revealed that PS was significantly increased in spring and summer, coinciding with highest light availability. Hard coral PS was significantly higher than soft coral PS. R, G, and POC were significantly higher in high temperature, low nutrient, summer compared to all other seasons and year-round higher for hard than soft corals. DOC fluxes were highly variable and not significantly different between genera or seasons. Significant positive correlations of PS were found with POC and DOC fluxes. This may be explained by the release of photosynthates as coral mucus entering both the POC and DOC pools of coral-surrounding seawater. Both PS and R correlated significantly and positively with G, confirming G as a light-enhanced and energy-demanding process. All fluxes were lower for soft corals than hard corals. Globally reported coral reef phase shifts from hard to soft coral dominance would consequently reduce essential processes such as primary production, organic matter recycling, and reef construction through calcification.

5)

Monitoring of coastal coral reefs near Dahab (Gulf of Aqaba, Red Sea) indicates local eutrophication as potential cause for benthic community phase-shifts

Naumann MS, **Bednarz VN**, Ferse SCA, Niggl W, Wild C

Under review at Environmental Monitoring and Assessment

Coral reef ecosystems fringing the coastline of Dahab (South Sinai, Egypt) have experienced increasing anthropogenic disturbance as an emergent international tourism destination. Previous reports covering tourism-related impacts on coastal environments, particularly mechanical damage and destructive fishing, have highlighted the vital necessity for regular ecosystem monitoring of coral reefs near Dahab. However, a continuous monitoring programme of permanent survey sites has not been established to date. Thus, this study conducted in situ monitoring surveys to investigate spatio-temporal variability of benthic reef communities and selected reef-associated herbivores along with reef health indicator organisms by revisiting three of the locally most frequented dive sites during expeditions in March 2010, September 2011 and February 2013. In addition, inorganic nutrient concentrations in reef-surrounding waters were determined to evaluate bottom-up effects of key environmental parameters on benthic reef community shifts in relation to grazer-induced top-down control. Findings revealed that from 2010 to 2013, live hard coral cover declined significantly by 12 % at the current-sheltered site Three Pools (TP), while showing negative trends for the Blue Hole (BH) and Lighthouse (LH) sites. Hard coral cover decline was significantly correlated to a substantial increase in turf algae cover (up to 57 % at TP) at all sites, replacing hard corals as dominant benthic space occupiers in 2013. These changes were correlated to ambient phosphate and ammonium concentrations that exhibited highest values (0.64 ± 0.07 μmol PO_4^{3-} L^{-1}, 1.05 ± 0.07 μmol NH_4^+ L^{-1}) at the degraded site TP and indicate a pronounced bottom-up control. In contrast, macroalgae appeared to respond to both bottom-up and top-down factors. Temporal variability measured in herbivorous reef fish stocks reflected seasonal impacts by local fisheries, with concomitant changes in macroalgal cover. These findings represent the first record of rapid, localised and bottom-up controlled phase-shifts in benthic reef communities near Dahab, and likely the entire Southern Gulf of Aqaba, underlining the necessity for efficient waste water management for coastal facilities in the region.

Supplementary information

Supplementary Tables for Chapters III, IV and VIII

Supplementary Table 3.S1. Fully crossed two-factor general linear model with N_2 fixation, net photosynthesis (P_{net}), respiration (R), gross photosynthesis (P_{gross}), and the ratio of P_{gross}:R as a function of substrate and season.

Parameter	Factor	df, $df_{residual}$	F	p
N_2 fixation	Season	6, 83	5.78	<0.001
	Substrate	3, 83	60.09	<0.001
	Season * Substrate	2, 77	299.37	<0.001
P_{gross}	Season	3, 79	12.68	<0.001
	Substrate	2, 79	63.64	<0.001
	Season * Substrate	5, 74	3.96	<0.001
R	Season	3, 89	18.00	<0.001
	Substrate	2, 89	156.27	<0.001
	Season * Substrate	6, 83	15.34	<0.001
P_{net}	Season	3, 80	6.65	<0.001
	Substrate	2, 80	210.64	<0.001
	Season * Substrate	5, 75	7.59	<0.001
P_{gross}:R	Season	3, 80	8.44	<0.001
	Substrate	2, 80	409.47	<0.001
	Season * Substrate	5, 75	6.52	<0.001

Supplementary Table 3.S2. Fully crossed three-factor general linear model with N_2 fixation as a function of substrate, season, and time of day (day vs. night).

	df, $df_{residual}$	F	p
Season	2, 122	106.12	<0.001
Substrate	2, 122	224.02	<0.001
Time of day	1, 122	6.86	<0.01
Season*Substrate	4, 114	6.22	<0.001
Season*Time of day	2, 114	9.28	<0.001
Substrate*Time of day	2, 114	10.39	<0.001
Season*Substrate*Time of day	4, 110	3.70	<0.01

Supplementary Table 4.S1. Summary of the background water parameters monitored during the four different seasons at 10 m water depth. Values are means of four weeks of measurements (± s.e.m.). For details on the sampling design for each variable see the Methods section.

Environmental variable	Winter (Feb)	Spring (Apr)	Summer (Sep)	Autumn (Nov)
Irradiance (PAR)	203 (6)	218 (24)	319 (9)	199 (5)
Temperature (°C)	22.5 (0.1)	22.8 (0.1)	27.5 (0.2)	25.2 (0.2)
Ammonium (µM)	0.32 (0.04)	0.46 (0.03)	0.11 (0.01)	0.28 (0.06)
Nitrate (µM)	0.34 (0.03)	0.44 (0.04)	0.04 (0.01)	0.13 (0.05)
Nitrite (µM)	0.37 (0.06)	0.12 (0.04)	0.02 (0.01)	0.02 (0.01)
Phosphate (µM)	0.11 (0.01)	0.10 (0.01)	0.04 (0.01)	0.04 (0.01)
POC (µM)	7.70 (0.21)	10.25 (1.48)	6.61 (0.61)	8.81 (0.49)
PN (µM)	0.93 (0.03)	1.27 (0.14)	0.74 (0.02)	0.87 (0.04)
DOC (µM)	76.62 (4.27)	71.95 (3.77)	84.71 (2.09)	80.81 (0.54)
Chlorophyll a (µg/L)	0.21 (0.01)	0.22 (0.02)	0.10 (0.01)	0.19 (0.02)
POC:PN ratio (mol/mol)	8.32 (0.19)	8.07 (0.59)	8.91 (0.63)	10.11 (0.48)
DIN:PO_4^{3-} ratio (mol/mol)	9.72 (0.96)	10.46 (1.01)	5.56 (1.64)	11.94 (2.15)

Supplementary Table 4.S2. Results of the PERMANOVA test for multivariate changes among the four seasons. Tested are differences in Irradiance (PAR), Temperature (Temp), Ammonium (NH_4^+), Nitrate (NO_3^-), Phosphate (PO_4^{3-}), Particulate organic C (POC), Particulate N (PN), Dissolved organic C (DOC), Chlorophyll *a* (chl *a*), POC:PN ratio (POC:PN), DIN:PO_4^{3-} ratio (DIN:PO_4^{3-}). See the Methods section for details on the test. ***, $P<0.001$.

	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Season	3	113	37.695	8.713	***	9916
Residual	12	52	4.326			
Total	15	165				

Supplementary Table 4.S3. Results of the two-way analyses of variance. Tested are the effects of the factors Season, Genus and their interaction on N_2 fixation, Net photosynthesis (Pn), Respiration (R), Gross photosynthesis (Pg), POC, DOC and TOC fluxes, Zooxanthellae density (Zoox), Chlorophyll a × cm^{-2} (chl *a*), Chlorophyll *a* × $zooxanthellae^{-1}$ (chl *a*/zoox). See the Methods section for details on the analyses. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; n.s., not significant.

Factor	N_2 fixation		Pn		R		Pg		POC fluxes		DOC fluxes		TOC fluxes		Zoox		chl *a*		chl *a*/zoox	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Season	49.6	***	5.2	***	5.5	**	3.2	*	2.8	*	0.8	n.s.	1.7	n.s.	47.3	***	18.6	***	51.8	***
Genus	8.5	***	4.3	**	1.4	n.s.	3.2	**	1.5	n.s.	0.8	n.s.	1.0	n.s.	6.1	**	2.7	n.s.	12.1	***
Season x Genus	2.9	**	2.5	**	3.0	**	1.9	*	1.3	n.s.	0.3	n.s.	0.3	n.s.	3.2	*	2.7	n.s.	12.7	***

Supplementary Table 4.S4. Results of *a posteriori* tests for the interaction term Season x Genus, if significant (cf. Supplementary Table 4.S3), looking at differences among seasons. Variables are N_2 fixation, Net photosynthesis (Pn), Respiration (R), Gross photosynthesis (Pg), Zooxanthellae density (Zoox), Chlorophyll *a* × $zooxanthellae^{-1}$ (chl *a*/zoox). See the Methods section for details on the analyses. *, $P<0.05$; **, $P<0.01$; n.a., not available.

Variable	Factor level	ACR				POC				STY				GON			
		WIN	SPR	SUM	AUT	WIN	SPR	SUM	AUT	WIN	SPR	SUM	AUT	WIN	SPR	SUM	AUT
N_2 fixation	WIN																
	SPR					*											
	SUM	**	**		**	**	*		**	**	**		**	**	**		**
	AUT													**	**		
Pn	WIN																
	SPR									**				**			
	SUM																
	AUT									*							
R	WIN		*										*				
	SPR												**				
	SUM												**	*	*		
	AUT																
Pg	WIN																
	SPR									**				*			
	SUM													*			
	AUT																
Zoox	SPR	n.a.		*	n.a.	n.a.			n.a.	n.a.		**	n.a.	n.a.		**	n.a.
	SUM	n.a.			n.a.	n.a.			n.a.	n.a.			n.a.	n.a.			n.a.
chl *a*/zoox	SPR	n.a.			n.a.	n.a.			n.a.	n.a.			n.a.	n.a.			n.a.
	SUM	n.a.			n.a.	n.a.	**		n.a.	n.a.	**		n.a.	n.a.	*		n.a.

Supplementary Table 4.S5. Results of *a posteriori* tests for the interaction term Season x Genus, if significant (cf. Supplementary Table 4.S3), looking at differences among coral genera. Variables are N_2 fixation, Net photosynthesis (Pn), Respiration (R), Gross photosynthesis (Pg), Zooxanthellae density (Zoox), Chlorophyll *a* × zooxanthellae^{-1} (chl *a*/zoox). See the Methods section for details on the analyses. *, $P<0.05$; **, $P<0.01$; n.a., not available.

Variable	Factor level	WIN				SPR				SUM				AUT			
		ACR	POC	STY	GON	ACR	POC	STY	GON	ACR	POC	STY	GON	ACR	POC	STY	GON
N_2 fixation	ACR																
	POC					**											
	STY					*											
	GON										*			**	**	**	
Pn	ACR			**													
	POC			**													
	STY																
	GON			*											*		
R	ACR		*														
	POC															*	
	STY																
	GON															*	
Pg	ACR			**	*												
	POC			*													
	STY																
	GON																
Zoox	ACR		n.a.	n.a.	n.a.										n.a.	n.a.	n.a.
	POC	n.a.		n.a.	n.a.									n.a.		n.a.	n.a.
	STY	n.a.	n.a.		n.a.									n.a.	n.a.		n.a.
	GON	n.a.	n.a.	n.a.		*	**	*						n.a.	n.a.	n.a.	
chl *a*/zoox	ACR		n.a.	n.a.	n.a.										n.a.	n.a.	n.a.
	POC	n.a.		n.a.	n.a.					**			*	n.a.		n.a.	n.a.
	STY	n.a.	n.a.		n.a.					**	**		**	n.a.	n.a.		n.a.
	GON	n.a.	n.a.	n.a.										n.a.	n.a.	n.a.	

Supplementary Table 4.S6. Results of *a posteriori* tests for the factor Season, if the interaction term was not significant (cf. Supplementary Table 4.S3). Variables are POC fluxes and Chlorophyll *a* × cm^{-2} (chl *a*). See the Methods section for details on the analyses. *, $P<0.05$; **, $P<0.01$; n.a., not available.

Variable	Factor level	WIN	SPR	SUM	AUT
POC fluxes	WIN			*	
	SPR				
	SUM				
	AUT				
chl *a*	SPR	n.a.		**	n.a.
	SUM	n.a.			n.a.

Supplementary Table 4.S7. Parameters used to build the C and N flux model. Parameters are either obtained from data sets generated by the present study (*) or calculated as described in the Supplementary Methods section. Values are reported as means ± standard deviation.

	Spring	**Summer**
*Daily gross photosynthesis (DPg, µmol C cm^{-2} d^{-1})	9.610 ± 1.695	9.235 ± 2.678
*Daily respiration (DR, µmol C cm^{-2} d^{-1})	6.643 ± 1.736	8.616 ± 2.547
Heterotrophic C uptake (HC, µmol C cm^{-2} d^{-1})	1.731 ± 0.404	2.148 ± 0.479
Total heterotrophic N uptake (µmol N cm^{-2} d^{-1})	0.247 ± 0.050	0.277 ± 0.053
Particulate organic N (PON) uptake (µmol N cm^{-2} d^{-1})	0.193 ± 0.046	0.217 ± 0.051
Dissolved free amino acids (DFAA) uptake (µmol N cm^{-2} d^{-1})	0.053 ± 0.004	0.059 ± 0.002
Urea uptake (µmol N cm^{-2} d^{-1})	0.001	0.001
*Total dissolved inorganic N (DIN) uptake (µmol N cm^{-2} d^{-1})	0.150 ± 0.022	0.018 ± 0.004
**Ammonium (NH_4^+) uptake* (µmol N cm^{-2} d^{-1})	0.068 ± 0.005	0.010 ± 0.001
**Nitrate (NO_3^-) uptake* (µmol N cm^{-2} d^{-1})	0.082 ± 0.016	0.008 ± 0.003
*Uptake from N_2 fixation (µmol N cm^{-2} d^{-1})	0.002 ± 0.001	0.010 ± 0.005
*Total organic C (TOC) release (µmol C cm^{-2} d^{-1})	1.168 ± 0.298	1.379 ± 0.262
Total organic N (TON) release (µmol N cm^{-2} d^{-1})	0.079 ± 0.023	0.087 ± 0.017
Zooxanthellae N demand (ZND) (µmol N cm^{-2} d^{-1})	0.096 ± 0.017	0.092 ± 0.027
Contribution of zooxanthellae-acquired C to animal	110 ± 18	86 ± 14

respiration (CZAR, %)		
Contribution of heterotrophically-acquired C to animal respiration (CHAR, %)	25	25
Contribution of zooxanthellae-acquired N (DIN) to ZND (CZND, %)	149 ± 22	18 ± 5
Contribution of zooxanthellae-acquired N (heterotrophic) to ZND (CZND, %)	59 ± 11	69 ± 12
Contribution of zooxanthellae-acquired N (N_2 fixation) to ZND (CZND, %)	2 ± 1	11 ± 5
Contribution of zooxanthellae-acquired N (total) to ZND (CZND, %)	209 ± 34	97 ± 21
Loss by organic C release of the total acquired C (LOC, %)	10 ± 3	12 ± 2
Loss by organic N release of the total acquired N (LON, %)	14 ± 4	28 ± 6

Supplementary Table 8.S1. Dissolved inorganic nutrients and light intensity over the course of the manipulation experiment. Values are means ± s.d. calculated from weekly sampling of inorganic nutrients (n = 4) and maximum daily averages (11:30 - 14:30) of light intensity (n = 22).

Environmental variable	
Ammonium (μM)	0.24 (0.13)
Phosphate (μM)	0.04 (0.01)
Nitrite (μM)	0.04 (0.02)
Nitrate (μM)	0.17 (0.11)
Light intensity (PAR)	297 (57)

Supplementary Table 8.S2. Results of three-factorial PERMANOVAs for P_N : R_D ratio, P_G, N_2 fixation, calcification and mucus release rates. Significant *p* values are in bold. ** Term has one or more empty cells.

Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
P_N : R_D ratio	Species (Sp)	1	307	307	21.5	**0.0002**	9922
	Treatment (Tr)	1	611	611	42.7	**0.0001**	9928
	Time (Ti)	2	244	122	8.5	**0.0009**	9947
	SpxTr	1	294	294	20.6	**0.0001**	9932
	SpxTi	2	745	373	26.1	**0.0001**	9961
	TrxTi**	1	572	572	40.0	**0.0001**	9926
	SpxTrxTi**	1	435	435	30.4	**0.0001**	9915
	Residuals	60	858	14			
	Total	69	5045				
P_G	Species (Sp)	1	215	215	6.3	**0.0160**	9906
	Treatment (Tr)	1	2575	2575	74.8	**0.0001**	9937
	Time (Ti)	2	2627	1314	38.2	**0.0001**	9951
	SpxTr	1	1411	1411	41.0	**0.0001**	9938
	SpxTi	2	1598	799	23.2	**0.0001**	9946
	TrxTi**	1	2041	2041	59.3	**0.0001**	9940
	SpxTrxTi**	1	1691	1691	49.1	**0.0001**	9916
	Residuals	60	2064	34			
	Total	69	15077				
Calcification	Species (Sp)	1	187	187	0.5	0.5647	9951
	Treatment (Tr)	1	166	166	0.5	0.6541	9937
	Time (Ti)	2	583	291	0.8	0.5291	9945
	SpxTr	1	85	85	0.2	0.8258	9929
	SpxTi	2	216	108	0.3	0.8655	9945
	TrxTi**	1	413	413	1.2	0.3570	9945
	SpxTrxTi**	1	389	389	1.1	0.3902	9934
	Residuals	35	12542	358			
	Total	44	14929				

Supplementary Table 8.2. continued.

Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
Mucus Release (TOC)	Species (Sp)	1	26	26	0.1	0.9402	9956
	Treatment (Tr)	1	907	907	2.0	0.1246	9952
	Time (Ti)	1	1713	1713	3.9	**0.0213**	9965
	SpxTr	1	334	334	0.8	0.5135	9958
	SpxTi	1	561	561	1.3	0.2921	9961
	TrxTi**	1	1066	1066	2.4	0.0870	9958
	SpxTrxTi**	1	351	351	0.8	0.4942	9954
	Residuals	44	19554	444			
	Total	51	25428				
Mucus Release (DOC)	Species (Sp)	1	54	54	0.2	0.8302	9948
	Treatment (Tr)	1	250	250	0.9	0.3523	9954
	Time (Ti)	1	842	842	3.1	0.0642	9941
	SpxTr	1	74	74	0.3	0.7494	9950
	SpxTi	1	200	200	0.7	0.4336	9932
	TrxTi**	1	508	508	1.9	0.1593	9944
	SpxTrxTi**	1	77	77	0.3	0.7413	9942
	Residuals	44	11808	268			
	Total	51	13948				
Mucus Release (POC)	Species (Sp)	1	317	317	1.5	0.2065	9942
	Treatment (Tr)	1	404	404	2.0	0.1469	9928
	Time (Ti)	1	1742	1742	8.5	**0.0021**	9947
	SpxTr	1	2716	2716	13.2	**0.0003**	9927
	SpxTi	1	1198	1198	5.8	**0.0094**	9948
	TrxTi**	1	1369	1369	6.6	**0.0065**	9940
	SpxTrxTi**	1	1072	1072	5.2	**0.0127**	9935
	Residuals	52	10715	206			
	Total	59	20180				

Supplementary Table 8.2. continued.

Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
N_2 fixation (light)	Species (Sp)	1	268	268	0.6	0.5319	9955
	Treatment (Tr)	1	251	251	0.5	0.5818	9948
	Time (Ti)	2	3309	1655	3.6	**0.0173**	9934
	SpxTr	1	184	184	0.4	0.7051	9957
	SpxTi	2	199	100	0.2	0.9328	9953
	TrxTi**	1	46	46	0.1	0.9593	9936
	SpxTrxTi**	1	76	76	0.2	0.8970	9940
	Residuals	52	23957	461			
	Total	61	28950				
N_2 fixation (dark)	Species (Sp)	1	396	396	2.2	0.1438	9933
	Treatment (Tr)	1	645	645	3.5	0.0578	9937
	Time (Ti)	2	3420	1710	9.3	**0.0004**	9954
	SpxTr	1	2	2	0.0	0.9919	9937
	SpxTi	2	97	48	0.3	0.8195	9962
	TrxTi**	1	1176	1176	6.4	**0.0121**	9936
	SpxTrxTi**	1	181	181	1.0	0.3380	9942
	Residuals	48	8798	183			
	Total	57	15189				

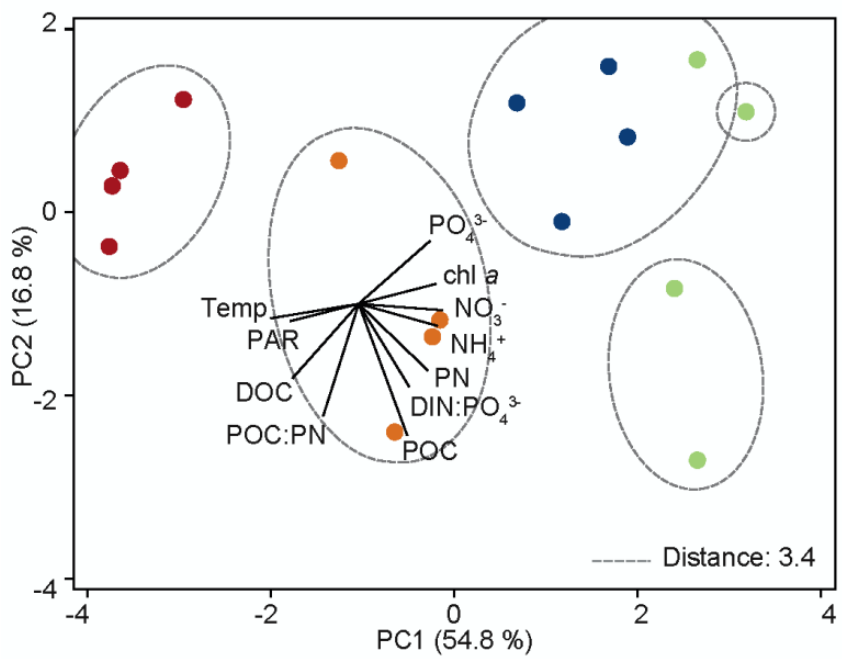
Supplementary Table 8.3. Results of two-factorial PERMANOVAs for *Symbiodinium* density, areal and *Symbiodinium* chlorophyll *a* content, picoeukaryotes and cyanobacteria growth, picoeukaryotes removal and microbial oxygen consumption. Significant *p* values are in bold.

Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
Symbiodinium density	Species (Sp)	1	10058	10058	16.2	**0.0001**	9947
	Treatment (Tr)	1	3505	3505	5.7	**0.0085**	9932
	SpxTr	1	3093	3093	5.0	**0.0132**	9938
	Residuals	18	11164	620			
	Total	21	29524				
Areal chlorophyll *a*	Species (Sp)	1	9714	9714	39.9	**0.0001**	9923
	Treatment (Tr)	1	7889	7889	32.4	**0.0001**	9944
	SpxTr	1	8024	8024	33.0	**0.0001**	9935
	Residuals	18	4379	243			
	Total	21	32412				
Symbiodinium chlorophyll *a*	Species (Sp)	1	1193	1193	1.7	0.1386	9960
	Treatment (Tr)	1	1878	1878	2.6	**0.0353**	9930
	SpxTr	1	2361	2361	3.3	**0.0135**	9949
	Residuals	18	12970	721			
	Total	21	18523				
Picoeukaryotes growth	Species (Sp)	1	51	51	0.4	0.8137	9926
	Treatment (Tr)	2	433	217	1.7	0.1195	9920
	SpxTr	2	108	54	0.4	0.8962	9931
	Residuals	30	3743	125			
	Total	35	4419				

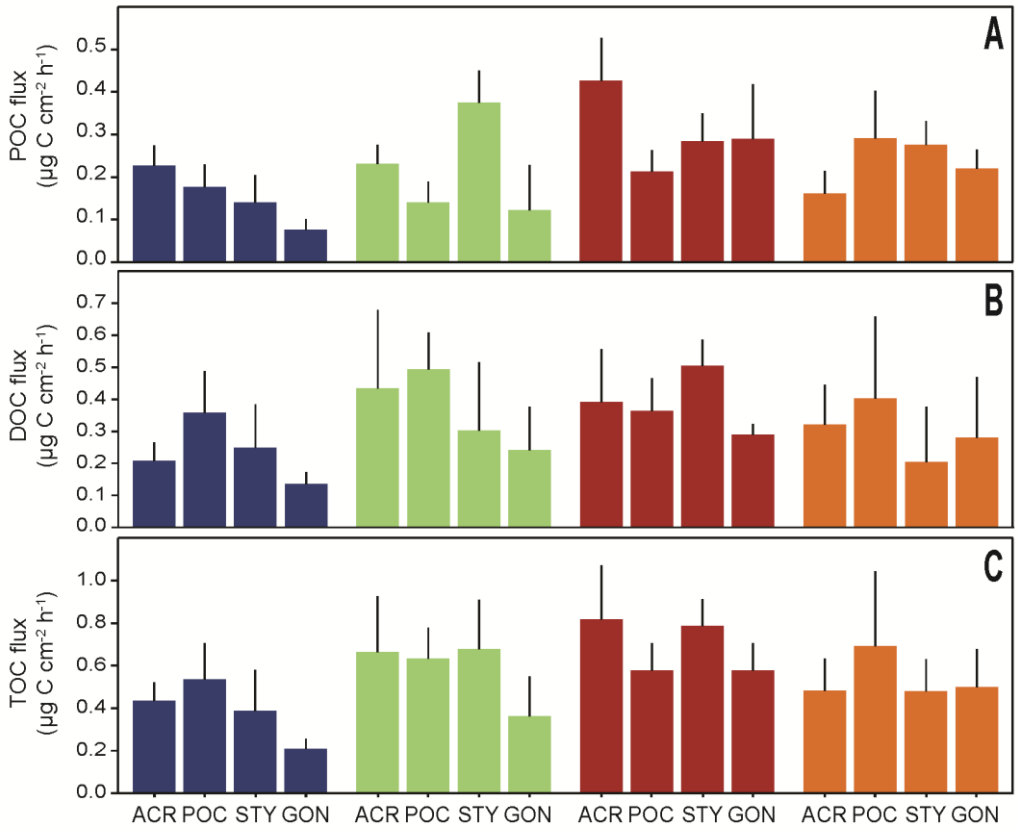
Supplementary Table 8.3. continued.

Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
Cyanobacteria growth	Species (Sp)	1	34	34	0.4	0.6717	9923
	Treatment (Tr)	2	248	124	1.5	0.2108	9946
	SpxTr	2	131	65	0.8	0.5466	9954
	Residuals	30	2474	82			
	Total	35	2916				
Picoeukaryotes removal	Species (Sp)	1	755	755	1.0	0.3850	9931
	Treatment (Tr)	1	5080	5080	6.6	**0.0003**	9940
	SpxTr	1	328	328	0.4	0.8188	9948
	Residuals	24	18590	775			
	Total	27	24726				
Microbial oxygen consumption	Species (Sp)	1	2463	2463	5.3	**0.0049**	9950
	Treatment (Tr)	1	4402	4402	9.4	**0.0002**	9950
	SpxTr	1	465	465	1.0	0.3720	9959
	Residuals	24	11212	467			
	Total	27	18631				

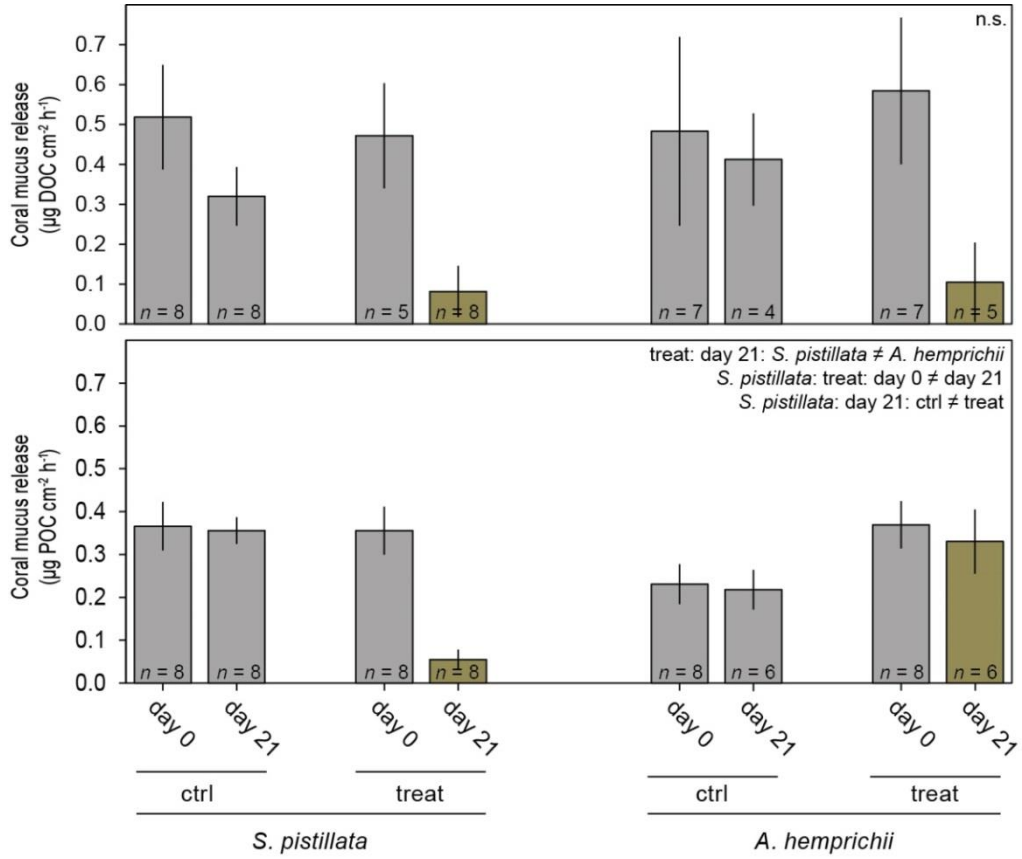
Supplementary Figures for Chapter IV and VIII



Supplementary Figure 4.S1. Principal Component Analysis (PCA) testing for multivariate change in environmental factors among the four seasons. Colours represent winter (blue), spring (green), summer (red), autumn (orange). Note that the per cent variation explained by the PCs is indicated on the axes and refers to the fraction of the total variance explained by Irradiance (PAR), Temperature (Temp), Ammonium (NH_4^+), Nitrate (NO_3^-), Phosphate (PO_4^{3-}), Particulate organic C (POC), Particulate N (PN), Dissolved organic C (DOC), Chlorophyll *a* (chl *a*), POC:PN ratio (POC:PN), DIN:PO_4^{3-} ratio (DIN:PO_4^{3-}).



Supplementary Figure 4.S2. Organic C fluxes of the four hard coral genera during the four seasons. Fluxes of particulate, dissolved and total organic C (POC, DOC and TOC, respectively) of *Acropora* (ACR), *Pocillopora* (POC), *Stylophora* (STY) and *Goniastrea* (GON) are presented here as means (n=6) ± s.e.m. For statistical differences see the Extended Data Table 3 and 6. Colours represent winter (blue), spring (green), summer (red) and autumn (orange).



Supplementary Figure 8.S1. POC and DOC fluxes of the coral nubbins at different times during the experiment. Values are means ± s.e. (*n* for each group indicated) and pairwise comparisons for significant factors ($p < 0.05$, PERMANOVA) are indicated on the top right corner of each graph (n.s., not significant). Grey bars, ambient temperature; green bars, ambient temperature after exposure to 34°C. Results of the PERMANOVAs are reported in table 2 and 3.

Supplementary Figure 8.S2. *Stylophora pistillata* after the exposure to 34 °C, with open polyps at night.

Supplementary Methods for Chapter IV

Calculations applied for the coral C and N flux model

Calculations of carbon (C) and nitrogen (N) fluxes presented by the model were primarily based on data sets generated by the present study. In case this option was not available, conservative assumptions were made in choosing established literature data, obtained for similar tropical scleractinian species from comparable regions/environmental conditions, whenever possible.

1.1. Per cent contribution of zooxanthellae-acquired N to zooxanthellae N demand (CZND)

Bioavailable N demand of zooxanthellae photosynthetic activity (ZND, μmol N cm^{-2} d^{-1}) was calculated from zooxanthellae daily gross photosynthesis (DPg) measured for the two respective seasons (spring and summer), assuming a conservative molar C:N ratio of 10 in the algal fraction[47], and a fraction of 0.1 of primary production fuelled by new N uptake[48]. For each particular uptake rate, the per cent contribution of zooxanthellae-acquired N (calculated as explained in section 1.4 and 1.5) to ZND (CZND) was derived by the equation: CZND[%]=(N uptake rate to the zooxanthellae)/ZND*100.

1.2. Daily gross photosynthesis, respiration and CZAR

Measured gross photosynthesis rates (Pg, μg O_2 cm^{-2} h^{-1}) were converted to daily gross photosynthesis rates (DPg) (μmol C cm^{-2} d^{-1}) for spring and summer, assuming 12 h of daylight and 1.1 as photosynthetic quotient[49]. Daily respiration (DR) was converted to μmol C cm^{-2} d^{-1} from the measured rates of dark respiration (R, μg O_2 cm^{-2} h^{-1}) for spring and summer, over a 24 h day cycle using 0.8 as respiratory quotient[49]. The per cent contribution of zooxanthellae-acquired C to daily animal respiration (CZAR) was calculated according to Muscatine *et al.*[49], using T=0.78 according to Tremblay *et al.*[50].

1.3. N uptake by N_2 fixation

N uptake rates derived from N_2 fixation (μmol N cm^{-2} d^{-1}) were calculated from our results for the two respective seasons using a theoretical molar ratio C_2H_4:N_2 of 3:1 without considering hydrogenase activity[21,51], but accounting for the 2 atoms of molecular N_2. For calculating CZND, the ratio of the N fixed by the N_2-fixing bacteria subsequently assimilated by the symbiotic

zooxanthellae was assumed equal to 0.97, as described for the first time by Foster *et al.*[52] for an association between a N_2 fixer and a eukaryotic unicellular alga.

1.4. C and N uptake by heterotrophy, and CHAR

Heterotrophic C uptake (HC, μmol C cm^{-2} d^{-1}) was calculated as HC=DR*25/100, assuming a conservative average 25% contribution of heterotrophically-acquired C to animal respiration (CHAR)[11,53,54] for spring and summer, resulting from relatively low and constant *in situ* feeding rates on low year-round particulate organic C (POC) and N (PON) concentrations in reef-surrounding waters of the Gulf of Aqaba (cf. Supplementary Table 4.S1). The assumption of constant seasonal feeding was derived from non-variable seasonal observations of micro- and mesozooplankton depletion in local coral reef-overlying waters[55], and from the locally constant, yet relatively insignificant, nanoplankton biomass[56,57], which is accepted among pico- and nanoparticles as the most important contribution to C (84-94%) and N (52-85%) ingested by corals[58]. Heterotrophic PON uptake (μmol N cm^{-2} d^{-1}) was derived from heterotrophic C uptake rates by applying local *in situ* POC:PN ratios (cf. Supplementary Table 4.S1) and an average N assimilation efficiency[59-63] of 90%. A fraction of 0.2 of the calculated flux was assumed to be subsequently transferred from the host to the zooxanthellae[63-65], and thus regarded relevant for zooxanthellae N demand and CZND calculations. Finally, total heterotrophic N uptake (μmol N cm^{-2} d^{-1}) was calculated for spring and summer as the sum of PON and dissolved organic N (DON) uptake, where DON uptake rate = dissolved free amino acids (DFAA) + urea uptake rates. Each respective DON uptake rate was calculated as described here below.

1.4.1. Dissolved free amino acids (DFAA)

Active carrier-mediated DFAA uptake at *in situ* DFAA concentrations was calculated applying Michaelis-Menten kinetics ($V = (V_{max}*[DFAA])/(K+[DFAA])$) according to Grover *et al.*[66], where V is DFAA uptake rate (nmol N cm^{-2} h^{-1}), V_{max} is maximum DFAA uptake rate (i.e. 7.52 nmol N cm^{-2} h^{-1}), [DFAA] is *in situ* DFAA concentration (μmol l^{-1}) and K is DFAA concentration at half-maximal uptake rate (i.e. 1.23 μmol l^{-1}). [DFAA] was calculated by converting seasonal *in situ* DOC concentrations (cf. Supplementary Table 4.S1) to DON concentrations applying DOC:DON=14[67] and assuming DFAA=0.1*DON[68]. A fraction of 0.33 of the calculated flux was assumed to be taken up by the zooxanthellae rather than by the host[66], and thus regarded relevant for zooxanthellae N demand and CZND calculations.

1.4.2. Urea

Active carrier-mediated urea uptake at *in situ* urea concentrations was calculated applying Michaelis-Menten kinetics ($V = (V_{max}*[Urea])/(K+[Urea])$) according to Grover *et al.*[69], where V is urea uptake rate (nmol N cm^{-2} h^{-1}), V_{max} is maximum urea uptake rate (i.e. 0.20 nmol N cm^{-2} h^{-1}), [Urea] is *in situ* urea concentration (µmol l^{-1}) and K is urea concentration at half-maximal uptake rate (i.e. 1.05 µmol l^{-1}), [Urea] = 0.3 µmol l^{-1} used as representative *in situ* concentration[68]. A fraction of 0.2 of the calculated flux was finally assumed to be uptaken by the zooxanthellae rather than by the host[69], and thus regarded relevant for zooxanthellae N demand and CZND calculations.

1.5. Dissolved inorganic N (DIN) uptake

Dissolved inorganic N (DIN) uptake (µmol N cm^{-2} d^{-1}) was calculated for spring and summer seasons as the sum of ammonium (NH_4^+) + nitrate (NO_3^-) uptake rates. Each respective uptake flux was calculated as described here below.

1.5.1. Ammonium (NH_4^+)

Active carrier-mediated NH_4^+ uptake at *in situ* NH_4^+ concentrations was calculated applying Michaelis-Menten kinetics ($V = (V_{max}*[NH_4^+])/(K+[NH_4^+])$) according to Muscatine & D'Elia[70], where V is NH_4^+ uptake rate (nmol N cm^{-2} h^{-1}), V_{max} is maximum NH_4^+ uptake rate (i.e., 6.39 and 2.59 nmol N cm^{-2} h^{-1} for spring and summer season, respectively), $[NH_4^+]$ is *in situ* NH_4^+ concentration (µmol l^{-1}) (cf. Supplementary Table 4.S7, Fig. 4.S1) and K is NH_4^+ concentration at half-maximal uptake rate[70] (i.e. 0.58 µmol l^{-1}). V_{max} original units (µmol N mg chl *a* h^{-1}) were converted to nmol N cm^{-2} h^{-1} using present study seasonal chlorophyll a cm^{-2} data (cf. Supplementary Fig. 4.S2). A fraction of 0.9 of the calculated flux was assumed to be taken up by the zooxanthellae rather than by the host, and thus regarded as relevant for zooxanthellae N demand[71,72] and CZND calculations.

1.5.2. Nitrate (NO_3^-)

Active carrier-mediated NO_3^- transport was calculated applying linear uptake kinetics ($V = 7.8273*[NO_3]$) at *in situ* NO_3^- concentrations according to Bythell[73], where V is NO_3^- uptake rate (nmol N cm^{-2} h^{-1}) and $[NO_3]$ is *in situ* concentration (nmol l^{-1}) (cf. Supplementary Table 4.7, Fig. 4.S1). As NO_3^- assimilation is exclusive to zooxanthellae[74-76], the calculated flux was assumed to be taken up at a ratio of 1 for CZND calculations.

1.6. Per cent loss by organic C (or N) release of the total acquired C (or N) (LOC or LON)

Total organic C (TOC) release was calculated as the sum of the measured POC and DOC release rates (Supplementary Fig. 4.S2) and calculated on a per day basis (μmol C cm^{-2} d^{-1}). The per cent loss by TOC release of the total acquired C (LOC) was calculated by the equation: LOC[%]=TOCrelease/(DPg+HC)*100. Total organic N (TON) release was calculated assuming POC:PON ratios of 11.0 in spring and of 13.4 in summer as measured by Naumann *et al.*[41] at the sampling site, and an average DOC:DON ratio of 18.6 obtained from literature[77-79]. The per cent loss by TON release of the total acquired N (LON) was calculated by the equation: LON[%]=TONrelease/∑(N uptake rates)*100.

Erklärung

Gemäß §6 der Promotionsordnung der Universität Bremen füe die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche vom 14. März 2007 versichere ich, dass die vorliegende Arbeit mit dem Titel,

„Benthic dinitrogen fixation in a Northern Red Sea coral reef under seasonally changing environmental conditions"

1. ohne unerlaubte fremde Hilfe selbstständig verfasst und geschrieben wurde
2. keine anderen als die angegebenen Quellen und hilfsmittel benutzt wurden
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht wurden
4. es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt.

Bremen, 30. Oktober 2014

Vanessa Nomi Bednarz