Dinitrogen fixation in coral reef ecosystems facing climate change

A dissertation by

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To my family

Summary

Biological dinitrogen (N_2) fixation exerts important control on the ocean's nitrogen (N) inventory and primary productivity. Among marine ecosystems, coral reefs are notable for their very high productivity in extremely oligotrophic waters, where N_2 fixation has long been thought to satisfy much of the demand for new N. Furthermore, N_2 fixation in coral reef ecosystems may importantly contribute to global marine N inputs. However, because of large uncertainty in (i) the distribution of N_2 fixation in coral reefs and (ii) the tightness of the coupling between N_2 fixation and primary production, the ecological significance of N_2 fixation in coral reef ecosystems is still not resolved. The core of this thesis describes and discusses three different aspects of N_2 fixation in coral reefs. The first part of the thesis, through a series of interconnected studies, focuses on the relationship between N_2 fixation and primary productivity in the water column and in key benthic organisms at a fringing coral reef in the northern Red Sea (Gulf of Aqaba). This site harbours some of the northernmost warm water coral reefs on Earth. The characteristic seasonality of the sampling location allowed an investigation of the contribution of N_2 fixing prokaryotes (diazotrophs) to changing N requirements of the primary producers in response to pronounced seasonal environmental variations. Findings reveal that the reef water and its planktonic community contributed only marginally to the input of fixed N into the reef ecosystem. Concurrently, benthic N_2 fixation was conspicuous, and the rule rather than the exception in all benthic substrates investigated. Rates of N_2 fixation varied greatly between different substrates. Microbial mats, turf algae, 'bare' hard substrates, and 'bare' sediment contributed most fixed N to the reef, while soft corals showed the lowest N_2 fixation activity. N_2 fixation rates were significantly higher in summer, when nutrient availability was lowest and water temperature and light intensity highest. This indicates an important role of diazotrophy in sustaining the N demand of reef primary producers during the nutrient-depleted season. Furthermore, the susceptibility of diazotrophs to the varying environmental conditions suggests that disturbances resulting from human activities will also likely determine changes in the extent of N_2 fixation on reefs. In this context, in the second part of this thesis, results of two different manipulative experiments are presented that showed that ocean warming and ocean acidification have a high potential to impact N_2 fixation in scleractinian corals. This part of the thesis focused on scleractinian coral holobionts (the cnidarian-dinoflagellate-diazotroph symbiosis) because of their role as primary reef ecosystem engineers. Results demonstrate that thermal stress impacted all holobiont members and suggest that physiological plasticity of coral-associated diazotrophs, by fixing additional N under high temperature, may help to maintain constant chlorophyll *a* levels and prevent the breakdown of the coral-algal symbiosis. However, these beneficial effects may be counterbalanced by the impact of the ongoing increase in dissolved CO_2 levels (i.e., ocean acidification). A decrease in coral host calcification resulted in decreased N_2 fixation activity, since these two processes may compete for energy in the holobiont. Overall, these results contribute to the understanding of the role that coral-associated diazotrophs play in the holobiont under normal and stressful conditions. Finally, in the third part of this thesis, the relative contribution of all typical

reef-associated habitats to primary production and N_2 fixation of the entire ecosystem is discussed using budget calculations and a geographic information system approach. Results show that high coral-cover areas of the reef, because of their structural complexity, were not only important for their primary productivity but also for their associated N_2 fixation, with areal rates competing with those of flat sandy areas. Overall, N_2 fixation was highly susceptible to environmental changes, with 4-fold areal rates in all reef habitats during the nutrient-depleted season, which provided ca. 20% of the N needed for net primary production. Finally, global extrapolations confirm that coral reef ecosystems are among the benthic communities contributing most to marine fixed N inputs. In conclusion, this thesis demonstrates the importance and susceptibility of N_2 fixation in coral reef ecosystems. Still, large uncertainties remain in the potential responses to climate change, requiring further assessments. It is clear, however, that the tight coupling between N_2 fixation and reef primary productivity will determine feedback loops where perturbations of N_2 fixation will have repercussions in the carbon cycle, and vice versa.

Zusammenfassung

Biologische Stickstofffixierung (N_2-Fixierung oder Diazotrophie) trägt wesentlich für den Gehalt an Stickstoff (N) und damit für die Primärproduktion in den Weltmeeren bei. Korallenriffe zählen zu den produktivsten marinen Ökosystemen, obwohl sie von extrem nährstoffarmen Gewässern umgeben sind. N_2-Fixierung in Korallenriffen wurde daher seit langem für einen Großteil des Eintrags an neuem N verantwortlich gemacht und könnte zudem entscheidend zum globalen Eintrag von N in die Weltmeere beitragen. Aufgrund der vorherrschenden Ungewissheit bezüglich (i) der Verbreitung von N_2-Fixierung in Korallenriffen und (ii) dem unmittelbaren Zusammenhang zwischen N_2-Fixierung und Primärproduktion, ist die ökologische Bedeutung der N_2-Fixierung in Korallenriffökosystemen noch nicht verstanden. Der Hauptteil dieser Dissertation beschreibt und diskutiert daher drei verschiedene Aspekte der N_2-Fixierung in Korallenriffen. Der erste Teil der Arbeit behandelt, in einer Reihe miteinander verbundener Studien, die Beziehung zwischen N_2-Fixierung und Primärproduktion in der Wassersäule und in benthischen Schlüsselorganismen eines Korallenriffs des nördlichen Roten Meers (Golf von Aqaba). In dieser Region kommen einige der nördlichsten Warmwasserkorallenriffe der Erde vor. Die charakteristische regionale Saisonalität im Untersuchungsgebiet ermöglichte Studien zum Beitrag von N_2-fixierenden (diazotrophen) Prokaryoten zum spezifischen N Bedarf von Primärproduzenten als Reaktion auf ausgeprägt variable Umweltbedingungen. Die Ergebnisse dieser Studien zeigen, dass die Planktongemeinschaft des Riffwassers einen nur geringfügigen Beitrag zur N_2-Fixierung im Riffökosystem leistet. Im Gegensatz dazu war die benthische N_2-Fixierung auffällig und eher die Regel als Ausnahme in allen untersuchten Substraten. N_2-Fixierungsraten variierten stark zwischen den verschiedenen Substraten. Mikrobielle Matten, Turf-Algen, spärlich besiedelte Hartsubstrate und Sedimente trugen vorrangig zur N_2-Fixierung im Riff bei, während Weichkorallen die niedrigste N_2-Fixierungsaktivität zeigten. N_2-Fixierungsraten waren signifikant höher im Sommer, wenn die Nährstoffverfügbarkeit am niedrigsten und die Wassertemperatur und Lichtintensität am höchsten waren. Dies weist auf die wichtige Rolle der Diazotrophie hin bezüglich der Deckung des N Bedarfs der Primärproduzenten während des nährstoffarmen Sommers. Die Beeinflussung diazotropher Prokaryoten durch variable Umweltbedingungen deutet an, dass Ökosystemstörungen als Folge anthropogener Aktivitäten vermutlich auch Auswirkungen auf die Intensität der N_2-Fixierung im Riff haben könnten. In diesem Zusammenhang stellt der zweite Teil dieser Dissertation Ergebnisse zweier unterschiedlicher manipulativer Experimente vor, die zeigen, dass Ozeanerwärmung und Ozeanversauerung ein hohes Potenzial besitzen, die N_2-Fixierung in Steinkorallen zu beeinflussen. Dieser Teil der Arbeit befasst sich mit Steinkorallen als Holobionten (die Symbiose aus Nesseltier, Dinoflagellaten und diazotrophen Prokaryoten), die als Ökosystem-Ingenieure eine fundamentale Rolle im Riff einnehmen. Die Ergebnisse zeigen, dass Temperaturstress alle Mitglieder der Holobiont-Gemeinschaft beeinflusst, und dass die physiologische Plastizität von Korallen-assoziierten diazotrophen Prokaryoten, durch erhöhte N_2-Fixierung unter erhöhten Temperaturen dazu beitragen

kann, den Gehalt an Chlorophyll a konstant zu halten, und damit den Zusammenbruch der Korallen-Algen-Symbiose zu verhindern. Allerdings könnten diese positiven Auswirkungen durch den negativen Einfluss der anhaltenden Konzentrationszunahme an gelöstem CO_2 (Ozeanversauerung) ausgeglichen werden. Ein Rückgang der Korallenkalzifizierung resultierte in verringerter N_2-Fixierungsaktivität, da diese beiden Prozesse möglicherweise im Holobiont um Energie konkurrieren. Insgesamt tragen die Ergebnisse des zweiten Teils zum Verständnis der funktionalen Rolle bei, die Korallen-assoziierte diazotrophe Prokaryoten im Holobiont unter normalen und stressinduzierten Umweltbedingungen spielen. Schließlich wird im dritten Teil dieser Dissertation mit Hilfe von Budgetberechnungen und der Anwendung eines Geoinformationssystems die Primärproduktion und N_2-Fixierung verschiedener Riffareale sowie des gesamten Riffökosystems diskutiert. Die Ergebnisse zeigen, dass Riffareale mit hoher Korallenbedeckung aufgrund ihrer strukturellen Komplexität nicht nur bezüglich Primärproduktion, sondern auch in Bezug auf N_2-Fixierung mit Stoffflussraten von Riffsandflächen konkurrieren. Insgesamt war die gemessene N_2-Fixierung stark durch saisonal-variable Umweltbedingungen beeinflusst, und zeigte 4-fache Fixierungsraten in allen Riffbereichen während der nährstoffarmen Jahreszeit. Dies stellte ca. 20% des für die Nettoprimärproduktion benötigten N bereit. Weiterführende globale Hochrechnungen bestätigen, dass Korallenriffökosysteme zu den benthischen Lebensgemeinschaften zählen, die den grössten Beitrag zur marinen N_2-Fixierung leisten. Zusammenfassend verdeutlicht diese Dissertation die Bedeutung der N_2-Fixierung in Korallenriffökosystemen, aber auch deren Anfälligkeit auf Umweltveränderungen. Dennoch bleiben große Wissenslücken bezüglich möglicher Reaktionen auf den globalen Klimawandel, welche weitere Untersuchungen erforderlich machen. Klar ist jedoch, dass die enge Kopplung zwischen N_2-Fixierung und Primärproduktion in Korallenriffen Rückkopplungsschleifen definiert, in denen Störungen der N_2-Fixierung Auswirkungen auf den Kohlenstoffkreislauf haben können, und umgekehrt.

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General Introduction

Motivation of the study

Coral reef ecosystems are among the most productive ecosystems on Earth, yet only thrive in tropical waters where ambient concentrations of dissolved nutrients are very low (Fig. I-1). Here, biological dinitrogen (N_2) fixation (diazotrophy) is thought to be important in replenishing the nitrogen reservoir, thus maintaining reef ecosystem productivity and its biological storage of carbon (D'Elia and Wiebe 1990; Capone 1996; O'Neil and Capone 2008). Since the 70's (Wiebe *et al.* 1975) many studies found high rates of N_2 fixation associated with several benthic substrates, ranging from sediments and cyanobacterial mats to macroalgae and scleractinian corals (reviewed by O'Neil and Capone 2008, and Cardini *et al.* 2014). Recently, there has been emergent recognition of the potential contribution of N_2-fixing symbioses, with the discovery of a cnidarian-dinoflagellate-diazotroph association (Lesser *et al.* 2004).

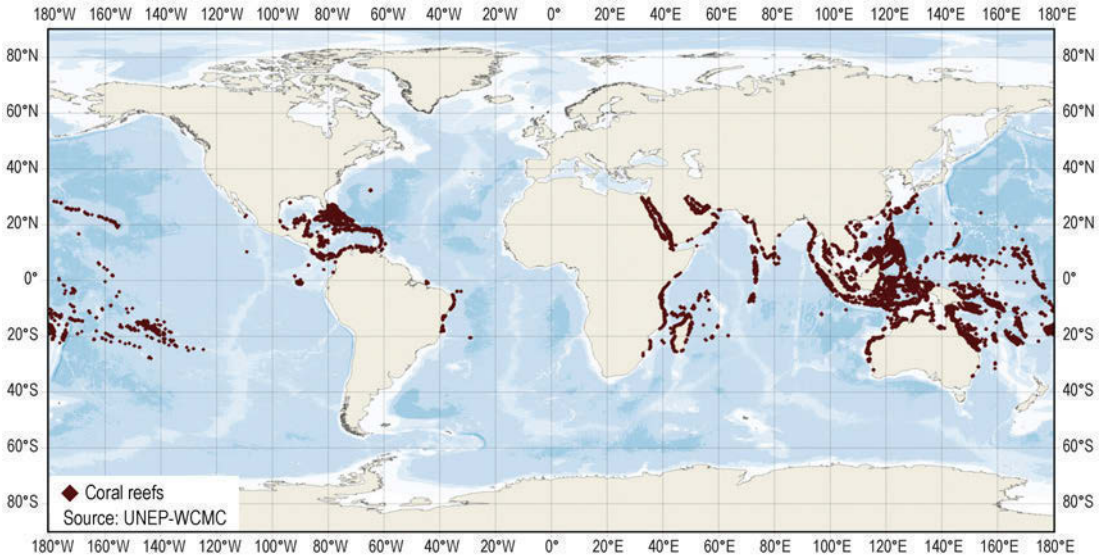

Figure I-1. Global distribution of coral reefs.

However large uncertainty remains, particularly with regards to the distribution of N_2 fixation in coral reefs and the tightness of the coupling between N_2 fixation and primary production, both at the organism and at the ecosystem level. Until now only scattered information is available, with studies focusing on single benthic groups (e.g. cyanobacterial mats) and measurements that often lack temporal resolution. Incubation procedures vary greatly among studies. Substrates potentially important for diazotrophy have been neglected. Thus, the ecological significance of N_2 fixation in coral reef ecosystems is still not resolved; while some studies attempted to assess the quantitative importance of N_2 fixation in coral reef ecosystems (Capone and Carpenter 1982; Larkum *et al.* 1988; Capone 1996), no systematic and comprehensive analyses considering the diversity of coral reef substrates have been performed so far that would allow for a synthesis. Moreover, a variety of different factors can affect the extent of N_2 fixation in an ecosystem (reviewed in Carpenter and Capone 2008). As coral reefs are increasingly exposed to anthropogenic climate change, the extent of N_2 fixation in

coral reefs may also be affected. Given the tight coupling between the nitrogen and the carbon cycle it is increasingly important to quantify N_2 fixation in coral reefs. Also, changing environmental conditions need to be considered if we want to understand how diazotrophy is contributing to the functioning of reef ecosystems facing climate change.

Objectives

This thesis addresses these gaps in knowledge through an investigation that addresses the following specific aims:

1. To quantify N_2 fixation with standardized methodology and with high temporal resolution in the water column and in all key benthic substrates and organisms that also contribute to primary production in a coral reef ecosystem.

2. To determine the potential effects of global anthropogenic climate change on N_2 fixation in the cnidarian–dinoflagellate–diazotroph symbiosis by means of manipulative experiments assessing the impact of two key global stressors: ocean warming and ocean acidification.

3. To evaluate the relative contribution of all key reef compartments to the production of fixed nitrogen and carbon in the reef environment, and to revise and discuss the role of N_2 fixation in biogeochemical cycling of coral reef ecosystems.

Approach

Fieldwork was carried out in 2013 at a fringing reef in front of the Marine Science Station (MSS, University of Jordan) in the northern Red Sea (Gulf of Aqaba) over all four seasons (Fig. I-2). At this high-latitude location, coral reefs are exposed to seasonally varying environmental conditions that result from thermal stratification of the water column in summer and deep-water mixing in winter (Silverman *et al.* 2007; Carlson *et al.* 2014). Thus, measurements in a high temporal resolution provide a solid basis for assessing the overall significance and range of N_2 fixation that may occur in coral reef environments. In addition, two manipulative experiments were conducted under controlled conditions to investigate the single effects of increased water temperature (ocean warming) and pCO_2 levels (ocean acidification) on N_2 fixation and other key metabolic processes in scleractinian corals. For measurements of N_2 fixation rates, the acetylene reduction assay (ARA, first described by Stewart *et al.* (1967) and Hardy *et al.* (1968)) was the method of choice. This method is based on the capacity of

nitrogenase (i.e., the enzyme responsible for N_2 fixation) to reduce C_2H_2 to C_2H_4 instead of N_2 to NH_3. Due to its simplicity, affordability and accuracy this indirect way of measuring N_2 fixation is by far the most commonly used technique, particularly in benthic habitats. Recently, modifications have been suggested to increase the sensitivity and accuracy of the method (Wilson *et al.* 2012). These modifications have been implemented in this thesis. Other methods are described in detail in each respective chapter.

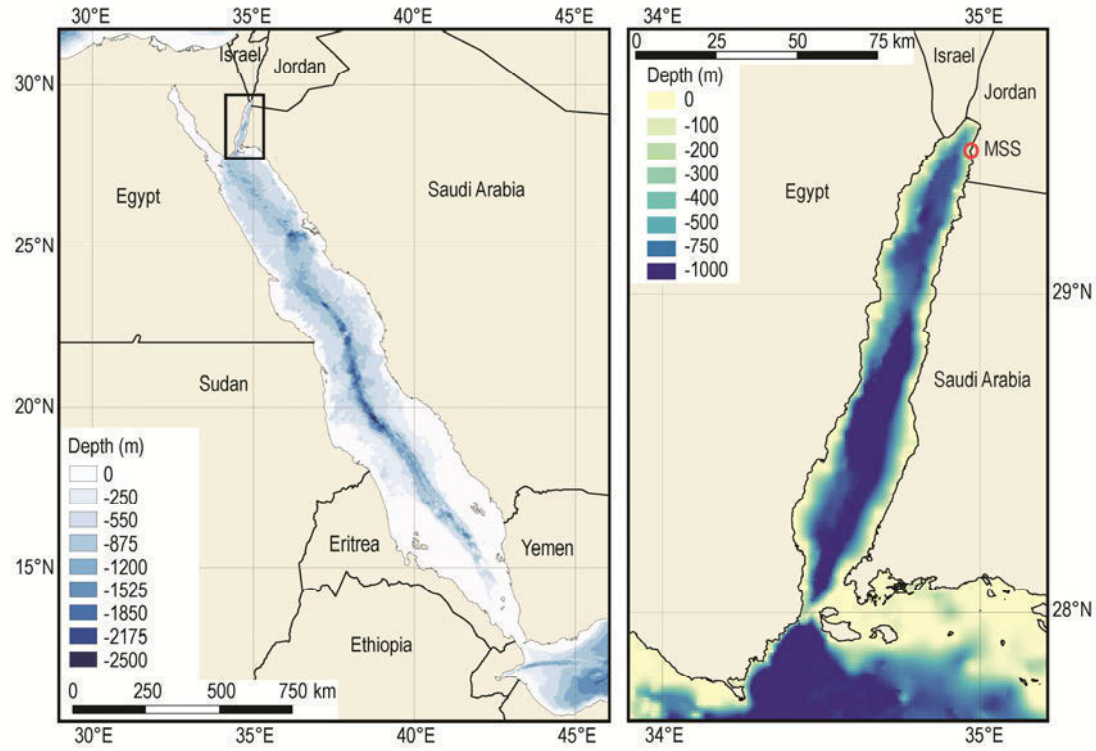

Figure I-2. Location of the study site, at the MSS (Aqaba, Jordan).

About the structure of this thesis

This thesis is composed of four general parts. Part I sets the stage for the subsequent experimental evidence. Within this part, **Chapter 1** summarizes the existing literature on benthic N_2 fixation in coral reefs and provides a descriptive overview of the symbioses between benthic reef organisms and diazotrophs. Additionally, known effects of human-induced environmental changes on the biological process of N_2 fixation are described. In this context, **Chapter 2** confirms that actual benthic community changes are occurring on coral reefs, providing visual evidence of structural habitat loss and community shift recorded over the last 37 years at the study site. Part II (**Chapters 3 to 7**) comprises a series of field investigations in which dinitrogen and carbon fixation by coral reef waters and benthic organisms are quantified in a seasonal resolution. In these Chapters the importance of N_2 fixation in sustaining primary productivity is considered with regards to the respective investigated category (water column, scleractinian hard corals, zooxanthellate soft corals, consolidated and

unconsolidated substrates). In Part III, the potential effect of ocean warming (**Chapter 8**) and acidification (**Chapter 9**) on the biological process of N_2 fixation in symbiotic scleractinian corals is investigated using a manipulative experimental approach under controlled conditions. Finally, in Part IV (**Chapter 10**) the relative contribution of all key investigated categories to the production of fixed nitrogen and carbon in the reef environment is evaluated, and the importance of N_2 fixation in biogeochemical cycling of coral reef ecosystems is revised and discussed.

List of Publications

This thesis is based on the scientific publications listed below, with authors contribution indicated.

Publication 1)

Cardini U, Bednarz VN, Foster RA, Wild C (2014) Benthic N_2 fixation in coral reefs and the potential effects of human-induced environmental change. *Ecology and Evolution* 4:1706-1727

The concept of this publication was developed by U. Cardini, V. Bednarz and C. Wild. Data analyses were carried out by U. Cardini. The manuscript was written by U. Cardini, with critical revision from all authors.

Publication 2)

Cardini U, van Hoytema N, Al-Rshaidat MMD, Schuhmacher H, Wild C, Naumann MS. Structural habitat loss and community shift recorded after 37 years on a Red Sea fringing reef (1976 - 2013). In preparation for *Coral Reefs*

This study was initiated by U. Cardini and M. Naumann. U. Cardini and N. van Hoytema analysed the photographic data and U. Cardini performed the digital image processing. U. Cardini and M. Naumann wrote the manuscript with input from all authors.

Publication 3)

van Hoytema N, **Cardini U**, Bednarz VN, Rix L, Naumann MS, Al-Horani F, Wild C. Effects of seasonality on planktonic primary production and dinitrogen fixation in a Red Sea coral reef. In preparation for *Limnology and Oceanography*

The concept of this publication was developed by N. van Hoytema, U. Cardini, V. Bednarz, and C. Wild. N. van Hoytema, U. Cardini, V. Bednarz and L. Rix conducted the fieldwork and sample processing. Data analyses were carried out by N. van Hoytema and U. Cardini. The manuscript was written by N. van Hoytema, with critical revision from all authors.

Publication 4)

Cardini U, Bednarz VN, Naumann MS, van Hoytema N, Rix L, Foster RA, Al-Rshaidat MMD, Wild C. Microbial dinitrogen fixation sustains high coral productivity in oligotrophic reef ecosystems. In preparation for *Scientific Reports*

This study was initiated by U. Cardini, V. Bednarz, N. van Hoytema and C. Wild. Fieldwork and sample processing was conducted by U. Cardini, V. Bednarz, N. van Hoytema, L. Rix. 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 analysed the data and wrote the manuscript with input from all authors.

Publication 5)

Bednarz VN, **Cardini U**, van Hoytema N, Al-Rshaidat MMD, Wild C. Seasonal variation of N_2 fixation and O_2 fluxes associated with two dominant zooxanthellate soft corals from the Northern Red Sea. Accepted for publication at *Marine Ecology Progress Series*

The concept of this publication was developed by V. Bednarz, U. Cardini, N. van Hoytema and C. Wild. V. Bednarz, U. Cardini and N. van Hoytema conducted the fieldwork and sample processing. Data analyses were carried out by V. Bednarz and U. Cardini. The manuscript was written by V. Bednarz, with critical revision from all authors.

Publication 6)

Rix L, Bednarz VN, **Cardini U**, van Hoytema N, Al-Horani F, Wild C, Naumann MS. Seasonality in dinitrogen fixation and primary productivity by coral reef framework substrates from the northern Red Sea. Under review at *Marine Ecology Progress Series*

This study was initiated by U. Cardini, V. Bednarz, N. van Hoytema and C. Wild. Fieldwork and sample processing was conducted by L. Rix, V. Bednarz, U. Cardini and N. van Hoytema. L. Rix analysed the data and wrote the manuscript with input from all authors.

Publication 7)

Bednarz VN, van Hoytema N, **Cardini U**, Naumann MS, Al-Rshaidat MMD, Wild C. Dinitrogen fixation and primary productivity by carbonate and silicate reef sand communities of the Northern Red Sea. Under review at *Marine Ecology Progress Series*

The concept of this publication was developed by V. Bednarz, U. Cardini, N. van Hoytema and C. Wild. V. Bednarz, U. Cardini and N. van Hoytema conducted the fieldwork and sample processing. V. Bednarz carried out the data analyses and wrote the manuscript, with critical revision from all authors.

Publication 8)

Cardini U, van Hoytema N, Bednarz VN, Rix L, Foster RA, Al-Rshaidat MMD, Wild C. The resilience of the coral holobiont to ocean warming depends on the cumulative response of its members. In preparation for *Global Change Biology*

This study was initiated by U. Cardini and C. Wild. The experimental work and sample processing was conducted by U. Cardini, V. Bednarz, N. van Hoytema and L. Rix. R. Foster assisted in the design of the acetylene reduction assays. U. Cardini analysed the data and wrote the manuscript with input from all authors.

Publication 9)

Rädecker N, Meyer FW, Bednarz VN, **Cardini U**, Wild C (2014) Ocean acidification rapidly reduces dinitrogen fixation associated with the hermatypic coral *Seriatopora hystrix*. *Marine Ecology Progress Series* 511:297-302

The concept of this publication was developed by N. Rädecker, F. Meyer, V. Bednarz, U. Cardini and C. Wild. N. Raedecker and F. Meyer conducted the experimental work and sample processing. U. Cardini and V. Bednarz assisted in the design of the acetylene reduction assays. U. Cardini supported during analysis and interpretation of data. N. Rädecker analysed the data and wrote the manuscript with critical revision from all authors.

Publication 10)

Cardini U, Bednarz VN, van Hoytema N, Rovere A, Naumann MS, Al-Rshaidat MMD, Wild C. Revising the role of biological dinitrogen fixation in biogeochemical cycling of coral reef ecosystems. In preparation for *Ecosystems*

This study was conceived by U. Cardini, V. Bednarz, N. van Hoytema and C. Wild. Fieldwork and sample processing were conducted by U. Cardini, V. Bednarz, and N. van Hoytema. A. Rovere assisted during geographic information system analyses. U. Cardini analysed the data and wrote the manuscript with input from all authors.

Related publications not included in this thesis:

Plass-Johnson JG, **Cardini U**, van Hoytema N, Bayraktarov E, Burghardt I, Naumann MS, Wild C (2015) Coral Bleaching. In: Armon RH, Hänninen O (eds) Environmental Indicators. Springer Netherlands, pp 117-146

Meyer FW, **Cardini U**, Wild C (2015) Ocean Acidification and Related Indicators. In: Armon RH, Hänninen O (eds) Environmental Indicators. Springer Netherlands, pp 723-742

Cardini U, Bednarz VN, van Hoytema N, Al-Rshaidat MMD, Wild C. Seasonal variations of carbon and dinitrogen fixation rates of a *Halophila stipulacea* seagrass meadow from the northern Red Sea. In preparation for *Limnology and Oceanography*

van Hoytema N, Bednarz VN, **Cardini U**, Naumann MS, Al-Horani F, Wild C. The influence of seasonality on benthic carbon fixation in a northern Red Sea coral reef. In preparation for *Marine Biology*

Bednarz VN, **Cardini U**, van Hoytema N, Naumann MS, Al-Rshaidat MMD, Wild C. The effect of light availability on dinitrogen fixation associated with scleractinian corals along a depth gradient in the northern Red Sea. In preparation for the *Journal of Experimental Biology*

van Hoytema N, Rix L, van Oevelen D, Bednarz VN, **Cardini U**, Naumann MS, Al-Horani F, Wild C. Inverse model analysis of seasonal carbon fluxes through a Red Sea fringing reef. In preparation for *Ecological Modeling*

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Part I

Setting the stage

Chapter 1
Benthic dinitrogen fixation in coral reefs: the potential effects of climate change

Abstract

Tropical coral reefs are among the most productive and diverse ecosystems, despite being surrounded by ocean waters where nutrients are in short supply. Benthic dinitrogen (N_2) fixation is a significant internal source of "new" nitrogen (N) in reef ecosystems, but related information appears to be sparse. Here, we review the current state (and gaps) of knowledge on N_2 fixation associated with coral reef organisms and their ecosystems. By summarizing the existing literature, we show that benthic N_2 fixation is an omnipresent process in tropical reef environments. Highest N_2 fixation rates are detected in reef-associated cyanobacterial mats and seagrass meadows, clearly showing the significance of these functional groups, if present, to the input of new N in reef ecosystems. Nonetheless, key benthic organisms such as hard corals also importantly contribute to benthic N_2 fixation in the reef. Given the usually high coral coverage of healthy reef systems, these results indicate that benthic symbiotic associations may be more important than previously thought. In fact, mutualisms between carbon (C) and N_2 fixers have likely evolved that may enable reef communities to mitigate N limitation. We then explore the potential effects of the increasing human interferences on the process of benthic reef N_2 fixation via changes in diazotrophic populations, enzymatic activities or availability of benthic substrates favourable to these microorganisms. Current knowledge indicates positive effects of ocean acidification, warming and deoxygenation and negative effects of increased ultraviolet radiation on the amount of N fixed in coral reefs. Eutrophication may either boost or suppress N_2 fixation, depending on the nutrient becoming limiting. Since N_2 fixation appears to play a fundamental role in nutrient-limited reef ecosystems, these assumptions need to be expanded and confirmed by future research efforts addressing the knowledge gaps identified in this review.

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Introduction

In the decades to come, life in the ocean will be confronted with a series of environmental conditions that have no parallel in human history (Harnik *et al.* 2012). Understanding and predicting the effects of human-induced climate change on marine ecosystems and the organisms within is therefore a current research priority (Garrard *et al.* 2012; Rees 2012; Salihoglu *et al.* 2012). Of particular concern are the effects of environmental change on marine microbes as microorganisms drive the elemental transformations of the biogeochemical cycles in the oceans and on land (Gruber 2011).

The marine nitrogen (N) cycle is one of the most important of all biogeochemical cycles, as N is an essential building block in all life forms. The N cycle significantly influences the cycles of other elements and particularly the carbon (C) cycle (Fig. 1.1), since N is considered the most limiting element for biological productivity in the open sea (Gruber 2008; Canfield *et al.* 2010). Research projects worldwide have focused on the N cycle and investigated the main consequences of human alteration as a result of the production and industrial use of synthetic nitrogen fertilizers (e.g. (Galloway *et al.* 1995; Galloway *et al.* 2003; Galloway *et al.* 2004). This resulted in an improved understanding of the consequences of the anthropogenic N problem (Galloway *et al.* 2004). On the contrary, less studied are the interactions of N with the C cycle and their consequences for the climate, particularly in the context of the increasing human interferences in the Earth system (Falkowski *et al.* 2000; Gruber and Galloway 2008). Indeed, understanding the N–C–climate interactions (Fig. 1.1) is becoming increasingly pressing as the release of carbon dioxide (CO_2) from the burning of fossil fuels is dramatically changing the world's climate (IPCC 2007).

The two main biological processes of the marine N cycle are N gain (i.e., dinitrogen (N_2) fixation, the conversion of N_2 to organic N) and N loss (i.e., denitrification, the conversion of nitrate to N_2, and Anammox, Anerobic Ammonium Oxidation). These are particularly important because of the inability of most marine organisms to use elemental N_2 (i.e., dissolved N gas, the most abundant chemical form). As a consequence, their balance determines the net biologically available N for the biosphere, and therefore marine productivity (Arp 2000; Gruber 2005,2008). Nevertheless, producing a balanced marine N budget has been difficult, with a large apparent deficit (~200 Tg N yr^{-1}) in the oceanic N_2 fixation rate compared to N loss (Mahaffey *et al.* 2005; Codispoti 2007; Deutsch *et al.* 2007), and it is still a matter of great debate (Großkopf *et al.* 2012).

Biological N_2 fixation can only be carried out by some prokaryotes, including a small but diverse group of bacteria and archaea commonly referred to as diazotrophs (Zehr *et al.* 2003; Kneip *et al.* 2007). The preferred ecological niche of diazotrophs was assumed to be largely limited to the open ocean oligotrophic gyres that are typically characterized by high light intensities, high O_2 concentrations and low N availabilities (Karl *et al.* 2002). However, recent research on the phylogenetic diversity and distributions of *nifH* (the functional gene which encodes for nitrogenase, the enzyme responsible for N_2 fixation) found N_2-fixing microorganisms throughout all marine environments, ranging from

deep-sea vents to highly productive shelf areas (Mehta *et al.* 2003; Zehr *et al.* 2003; Dekas *et al.* 2009; Farnelid *et al.* 2011; Fernandez *et al.* 2011; Hamersley *et al.* 2011).

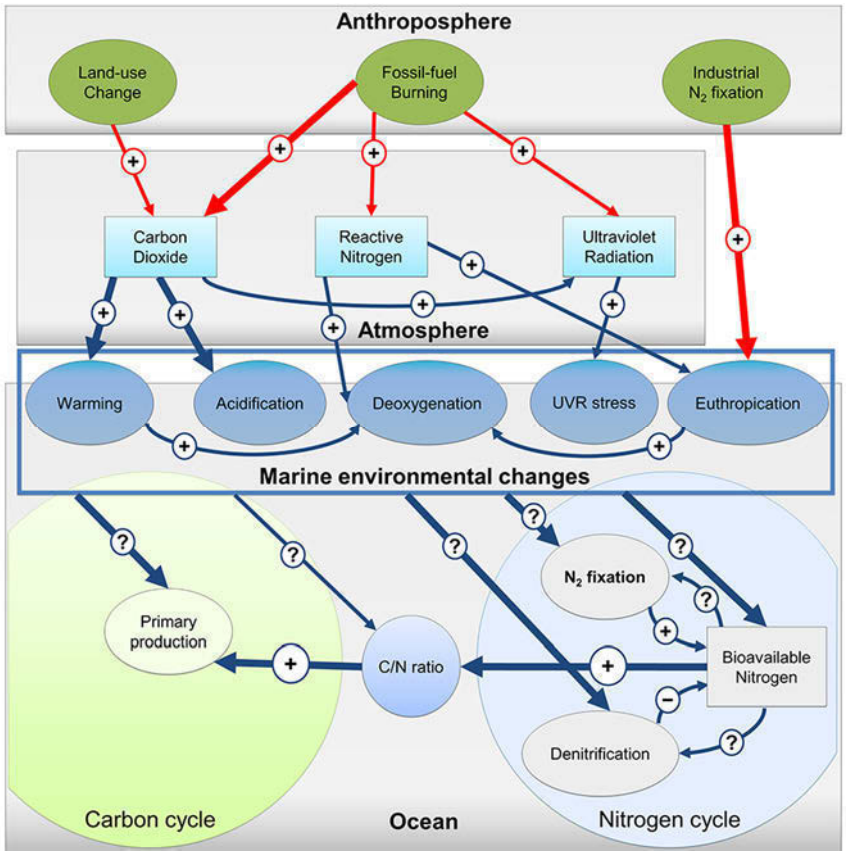

Figure 1.1. Nitrogen-carbon-climate interactions. Shown are the main interacting drivers during the Anthropocene. Signs indicate an increase (+) or a decrease (-) on the factor shown; (?) indicate an unknown impact. Colours of the arrow indicate direct anthropogenic impacts (red) or natural interactions (blue, many of which also modified by human influence). Strength of the interaction is expressed by the arrow thickness. Only selected interactions are represented. Adapted from Gruber and Galloway (2008).

Shallow coral reef environments are also recognized as major contributors of new N into the oceans, likely supporting a major fraction of total benthic N_2 fixation on a global scale (O'Neil and Capone 2008). However, these ecosystems are highly vulnerable and face an uncertain future as they are exposed to multiple increasing anthropogenic disturbances such as global warming, ocean acidification, increasing ultraviolet (UV) exposure, sea level rise, eutrophication, pollution, overfishing, and shoreline development (Hughes *et al.* 2010; Pandolfi *et al.* 2011; Frieler *et al.* 2013).

Global climate change and other anthropogenic pressures have the potential to not only alter the physiology of reef organisms directly (Hoegh-Guldberg *et al.* 2007; De'ath *et al.* 2009; Kleypas and Yates 2009), but also indirectly through impacts on reef-associated microorganisms (Vega Thurber *et al.* 2009; Meron *et al.* 2011; Witt *et al.* 2011). These invisible players are important drivers of coral

reefs (Barott and Rohwer 2012). In fact, as microorganisms are the fastest in reacting to disturbances and their responses are often non-linear, they are able to provide near real-time trajectories for a coral reef (Barott and Rohwer 2012). Therefore, their study is fundamental for understanding the functioning of the entire ecosystem in the face of climate change. Finally, as we come to understand the implications of the increasing anthropogenic pressure on coral reef environments, it is important that we evaluate impacts on major biogeochemical processes, and N_2 fixation is an obvious key ecological process that requires such evaluation.

Several publications recently attempted to review the current literature on N_2 fixation. However, these have been focused primarily on the open ocean (Karl *et al.* 2002; Mahaffey *et al.* 2005; Sohm *et al.* 2011a; Zehr 2011), on recent discoveries of previously unknown or less studied symbiotic associations with diazotrophs (Foster and O'Mullan 2008; Fiore *et al.* 2010), or on the *nifH* gene diversity and distribution (Zehr *et al.* 2003; Riemann *et al.* 2010), while only a few focused on coral reefs (Capone 1996; Carpenter and Capone 2008; O'Neil and Capone 2008). Moreover, these latter reports have mainly concentrated on the N cycle as a whole and particularly on the contribution of fixed N to the global ocean by coral reef habitats. However, recent findings stress the importance of specific associations between benthic reef organisms and N_2 fixers and furthermore identify the potential effects of climate change. Therefore, our primary foci are to review the present state of knowledge on benthic N_2 fixation in coral reefs and to provide a descriptive overview of the symbioses between benthic reef organisms and diazotrophs. Additionally, we will review the current knowledge on the effects of several anthropogenic impacts on the biological process of N_2 fixation. However, due to the lack of information available with regard to the effects of environmental conditions on benthic reef N_2 fixation, reference to the more studied pelagic realm will be given in the text when appropriate for comparison. Finally, we will provide a baseline upon which future coral reef research can build, suggesting some key research questions to be addressed and promising methodologies to be applied which may help to shed light on this crucial reef biogeochemical process.

N_2 fixation in coral reef ecosystems

Reef productivity

The study of relationships between C and nutrient fluxes is central to understanding material and energy fluxes in coral reefs, which ultimately set limits to metabolic performance of the ecosystem (Atkinson 2011). Although coral reefs show the highest rates of gross primary productivity worldwide, their existence is generally associated with waters that are very low in the nutrients necessary for primary production, akin to 'oases in a marine desert' (Hoegh-Guldberg 1999). This conundrum is generally known as the reef paradox, sometimes called the Darwin's paradox since he was the first to notice it. Remarkably different is their estimated net community production (i.e., the difference

between gross primary production and respiration), with a net gain (or loss) of matter within the system which is approximately zero (Gattuso *et al.* 1998).

The high biomass and gross productivity of these ecosystems is therefore explained by a tight internal recycling of matter that primarily occurs in the benthos (O'Neil and Capone 2008). Indeed, most of the organic matter produced on the reef is recycled and retained in living organisms or sediments within the reef system (Suzuki *et al.* 1995; Wild *et al.* 2004). Reef productivity cannot be sustained through the limited input of nutrients from the surrounding oceans, although sometimes these can be supplied by upwelling or internal tidal bores (Gattuso *et al.* 1998) as well as by nutrient advection or loading from land (Lapointe *et al.* 2004; Alongi and McKinnon 2005). Therefore, a thriving coral reef needs a finely tuned microbial-driven system to capture and recycle the nutrients necessary to support primary production (Garren and Azam 2012). On the other hand, if only regenerated nutrients were available, gross photosynthesis could not exceed respiration, unless a change in elemental ratios occurred: net growth and net export require the input of new N into the system (Szmant-Froelich 1983).

Benthic N_2 fixation as a primary N source

In the past, benthic N_2 fixation was considered the main source of 'new' N in the World's oceans (Capone and Carpenter 1982). However, actual estimates suggest a much greater contribution of pelagic N_2 fixation (a substantial fraction can be attributed to the colonial filamentous free-living cyanobacterium *Trichodesmium* spp.) compared to the amount of N that is fixed annually by benthic N_2 fixers (Gruber 2004). Recent research showed that diazotrophs in the smaller size fraction (< 10 µm cell diameter) are likely an equally important source of new N in the open ocean (Zehr *et al.* 2001; Mazard *et al.* 2004; Montoya *et al.* 2004; Needoba *et al.* 2007; Moisander *et al.* 2010). Moreover, several have identified that rates of N_2 fixation are often underestimated because of current methodological approaches (Mohr *et al.* 2010; Wilson *et al.* 2012). However benthic N_2 fixation estimates are based on old studies, mostly snapshots of particular benthic environments extrapolated to much larger areas. Therefore, a re-evaluation is necessary, since we are only beginning to understand the extent and importance of benthic marine N_2 fixation. In general, the seafloor hosts a wide diversity of geological and ecological settings supporting unique microbiological and faunal communities that might greatly contribute to the global input of fixed N (e.g. Dekas and Orphan (2011). In particular, benthic N_2 fixation assumes an overwhelming role in those ecosystems whose primary production is strongly N-limited and which are surrounded by highly N-depleted oceanic waters, such as coral reefs. Indeed, several coral reef studies observed export of N in the form of nitrate (NO_3^-), dissolved organic (DON) and particulate organic nitrogen (PON) in excess of inputs (Webb *et al.* 1975; Smith 1984; Suzuki and Casareto 2011), implying a source of fixed N from within the reef community, which can be attributed to N_2 fixation.

Introductions of bioavailable N through N_2 fixation can increase rates of primary production (Dugdale and Goering 1967), and low $\delta^{15}N$ signatures noted in several reef primary producers is consistent with

the hypothesis that much of the N in reef systems is derived from N_2 fixation (Yamamuro *et al.* 1995; Hilting *et al.* 2013). However, N_2 fixation in coral reef environments remains under-investigated and likely underestimated (O'Neil and Capone 2008).

Distribution and abundance of diazotrophs on coral reefs

Epibenthic diazotrophs

Epibenthic biofilms on solid surfaces are present everywhere in the aquatic environment. In particular, biofilms growing on living organisms may affect the fluxes of information, chemical signals, energy, nutrients, and matter across the host's body surface. Therefore, biofilms have an important ecological role in controlling the abiotic and biotic interactions of the host (Wahl *et al.* 2012).

In coral reef ecosystems, studies on benthic N_2 fixation studies have largely focused on microbial mats (Charpy-Roubaud *et al.* 2001; Steppe *et al.* 2001; Charpy-Roubaud and Larkum 2005; Charpy *et al.* 2007; Charpy *et al.* 2012a; Charpy *et al.* 2012b). These are dominated by cyanobacteria, which are found associated with sulphur bacteria and other microorganisms (Charpy *et al.* 2012a). They form flat, extensive mats, several millimeters thick on sand and limestone. These bacterial mats show the highest rates of N_2 fixation when compared to all the other main reef benthic components (Fig. 1.2). Charpy-Roubaud *et al.* (2001) found that N_2 fixation associated with reef lagoon sediments, limestone surfaces and particularly cyanobacterial mats could account for about 25 % of the N demand of benthic primary production in a coral atoll in French Polynesia. A follow up study investigated the reef rim at the same location and found similarly high areal rates largely associated with cyanobacterial mat communities accounting for about 28 % of N_2 fixation of the entire lagoon (Charpy-Roubaud and Larkum 2005).

N_2 fixation in seagrass meadows have also been extensively studied in tropical coral reef areas (Patriquin and Knowles 1972; McRoy *et al.* 1973; Capone *et al.* 1979; Capone and Taylor 1980; O'Donohue *et al.* 1991; Moriarty and O'Donohue 1993; Blackburn *et al.* 1994; Welsh 2000; Hamisi *et al.* 2009), showing rates comparable to those observed in cyanobacterial mats (Fig. 1.2). Although some studies mainly attributed the measured nitrogenase activity to the epiphytic cyanobacteria on the leaves, often high activities were found associated with the root systems and the rhizosphere sediments, where phototrophic organisms could be out-competed (Welsh 2000). Patriquin and Knowles (1972) argued that heterotrophic bacteria within rhizosphere sediments were providing most of the fixed N requirements in three different seagrass meadows from Barbados. Later observations demonstrated the importance of sulfate-reducing bacteria (SRBs) in rhizosphere N_2 fixation (Capone 1982; McGlathery *et al.* 1998), and that an appreciable fraction of the energy derived from sulfate reduction supported N_2 fixation (Welsh *et al.* 1996b; Welsh *et al.* 1996a; Welsh *et al.* 1996c; Nielsen *et al.* 2001).

N_2 fixation activity also occurs on limestone surfaces, coral rubble and coral skeletons (Fig. 1.2)(Crossland and Barnes 1976; Larkum 1988; Shashar *et al.* 1994a; Shashar *et al.* 1994b; Davey *et al.* 2008). These "bare" substrates are typically omnipresent on coral reefs, but their contribution to the total benthic cover is strongly dependent on the hydrodynamics and sedimentation rate. High rates of nitrogenase activity have been found in coral rubble from the Red Sea (Shashar *et al.* 1994b) and limestone substrates from the Great Barrier Reef (Larkum *et al.* 1988). Moreover, skeletons of coral which had undergone thermal bleaching showed high associated nitrogenase activity, with rates up to 30 times greater than those measured on live corals (Davey *et al.* 2008).

N_2 fixation has been further identified in bacterial epiphytes on benthic reef macroalgae (Capone *et al.* 1977; France *et al.* 1998; Koop *et al.* 2001) as well as associated with algal turfs (Fig. 1.2)(Williams and Carpenter 1997,1998). N_2-fixing cyanobacteria were among the dominant active members of the microbial community associated with a red alga of the broadly distributed genus *Laurencia* (de Oliveira *et al.* 2012). Active N_2 fixers are associated with members of the green algal genera *Caulerpa* (Williams *et al.* 1985; Chisholm and Moulin 2003) and *Codium* (Rosenberg and Paerl 1981). Both these algae are common on sandy and rocky reef substrates in intertidal and subtidal zones of tropical and subtropical coastal waters throughout the world. *Caulerpa taxifolia* appears to enhance N_2 fixation by releasing photosynthetic products into the rhizosphere (Chisholm and Moulin 2003). The excreted organic C, consumed by fermenting bacteria, creates substrate and strong reducing conditions that are favourable to N_2 fixation by SRBs. This process enhances organic matter turnover and nutrient supply to the alga's rhizoids, assisting this species to proliferate upon refractory organic sediments in low-nutrient seawater (Chisholm and Moulin 2003).

Active diazotrophs are also found associated with the ubiquitous reef carbonate sediments (Wilkinson *et al.* 1984; Corredor and Morell 1985; O'Neil and Capone 1989; Capone *et al.* 1992; Miyajima *et al.* 2001; Hewson and Fuhrman 2006; Werner *et al.* 2008), where N_2 fixation can account for substantial N flow into the system (Capone *et al.* 1992). In fact, rates of N_2 fixation in non-vegetated reef sediments are one order of magnitude lower than in cyanobacterial mats (Fig. 1.2)(Burris 1976; Iizumi and Yamamuro 2000; Bauer *et al.* 2008), but, when extrapolated over the entire reef area covered by mobile substrates, they can make a significant contribution to the overall coral reef N budget (Capone 1996; O'Neil and Capone 2008).

Symbiotic associations

Interest in marine microbial symbioses is growing rapidly because of the increasing awareness of the vast range of animal-bacterial interactions that is fundamentally altering our understanding of animal biology (McFall-Ngai *et al.* 2013). Symbioses have the potential to increase the fitness of the host and are implicated in its metabolism and growth, chemical defense production, as well as its susceptibility to biotic and abiotic stressors (Erwin *et al.* 2012). Specifically, several benthic organisms have coevolved nutritional mutualisms with diazotrophic bacteria in N-limited environments such as coral reefs (Fiore *et al.* 2010).

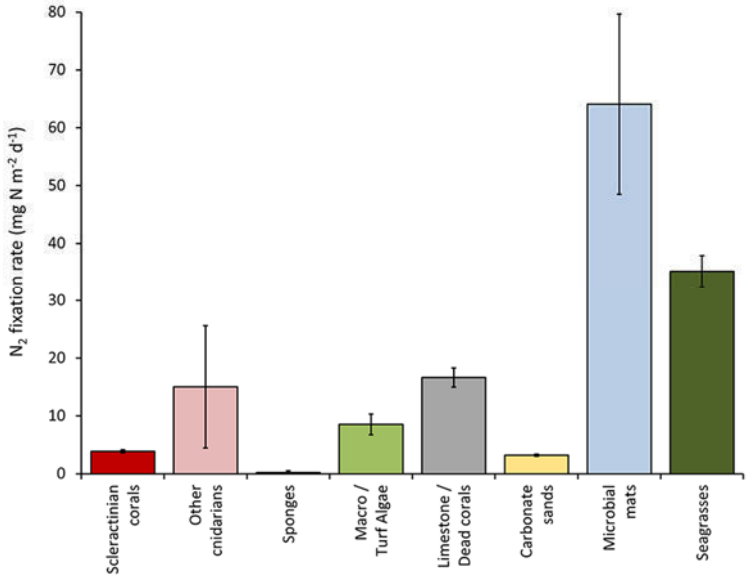

Figure 1.2. Contribution of the main benthic coral reef components to the input of new N in the reef via N_2 fixation. Rates (Average ± SE) were obtained from the available studies which reported nitrogenase activity associated with benthic reef organisms and substrates normalized to surface area. A list of the literature used is available in Appendix I - Table 1.

Symbiotic cyanobacteria and bacteria are found in almost all marine sponges (Carpenter and Foster 2002; Thacker 2005; Webster and Taylor 2012) where the processes of N_2 fixation, nitrification, denitrification and Anammox were all reported to occur (Wilkinson and Fay 1979; Diaz and Ward 1997; Wilkinson 1999; Mohamed *et al.* 2008; Hoffmann *et al.* 2009; Mohamed *et al.* 2009; Schläppy *et al.* 2010). However, the study of the ecological significance of diazotrophic symbionts in sponges and their contribution to the reef N budget has proven difficult (Wilkinson 1999). Rates shown in Fig. 1.2 are taken from the only study, to our knowledge, which has reported values normalized to surface area (Shashar *et al.* 1994b). However, N_2 fixation rates were measured by Shashar *et al.* (1994b) using the Acetylene Reduction Assay, and following studies suggested that this method is underestimating N_2 fixation in sponges (Wilkinson 1999). A later study by Mohamed *et al.* (2008) investigated the diversity and expression of N_2 fixation genes in bacterial symbionts of four different sponge species from Key Largo, Florida, and suggested that provision of fixed N via the symbionts benefits host sponges in nutrient-limited reef environments. N_2 fixation by sponge symbionts could therefore be a potentially important source of new N to the reef environment; an assumption that requires further investigation. The correlation between $\delta^{15}N$ signatures of different sponges and the composition of the associated microbial communities (Weisz *et al.* 2007), together with the evidence of stability of the sponge microbiota over large seasonal shifts (Erwin *et al.* 2012), strengthen the hypothesis of stable and host-specific associations between bacteria and reef sponges.

Besides sponges, diazotrophs are also found associated with corals (Rohwer *et al.* 2001; Frias-Lopez *et al.* 2002; Rohwer *et al.* 2002; Lema *et al.* 2012; Lema *et al.* 2014) and N_2 fixation activity has been

measured in live hard coral tissues (Williams *et al.* 1987; Shashar *et al.* 1994a; Shashar *et al.* 1994b). N_2 fixation rates detected in corals are comparable to those measured in reef carbonate sediments (Fig. 1.2). This suggests that their contribution to the input of new N in reef ecosystems may also prove very important when extrapolated to the entire reef area covered by hard substrates.

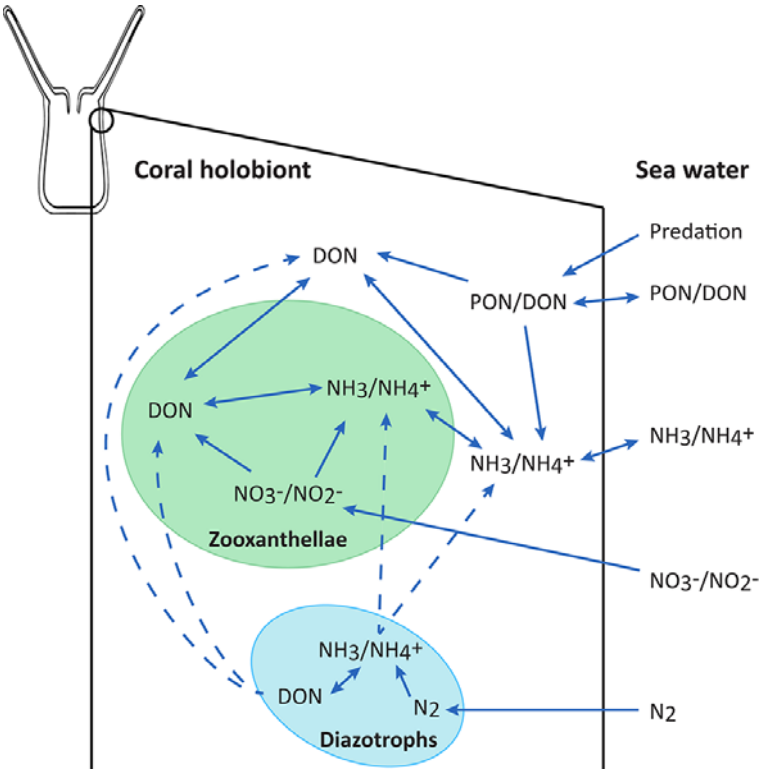

Figure 1.3. Schematic illustration of the N cycle in the coral holobiont. Solid lines represent nutrient transfer and pathways that have been shown to occur, while dashed lines represent hypothetical fluxes.

Endolithic cyanobacteria are common organisms inhabiting the skeleton of scleractinian corals, where they often occur as discrete bands at various depths in the skeletal matrix below the living coral tissue (Le Campion-Alsumard *et al.* 1995; Fine *et al.* 2005; Ralph *et al.* 2007) and can be important in providing nutrients to the coral (Ferrer and Szmant 1988). Recently, evidence of endosymbiosis with N_2-fixing cyanobacteria in corals was found in the colonial stony coral *Montastraea cavernosa* (Lesser *et al.* 2004; Lesser *et al.* 2007). Similar symbionts have also been observed in Acroporid corals from the Great Barrier Reef (Kvennefors and Roff 2009), thereby suggesting that this association may be widespread. However, recent studies on different coral species using molecular approaches targeting the *nifH* gene have revealed that diverse diazotrophic assemblages occur associated with coral tissues (Olson *et al.* 2009; Lema *et al.* 2012; Lema *et al.* 2014), and that *nifH* containing cyanobacteria often represent only a minor fraction of these communities (Lema *et al.* 2012; Lema *et al.* 2014). Diazotrophic assemblages in the coral tissue were species-specific, with the dominant phylotypes closely related to the bacterial group Rhizobia. Rhizobia species are common soil bacterial symbionts, residing in root nodules of legumes, and function as N_2 fixers for their host plants. This group was

consistently dominant in *Acropora millepora* at different locations throughout the year, suggesting a key functional role also in the coral (Lema *et al.* 2014).

Symbiotic corals have evolved a complex internal N cycle which allows them to thrive in N-limited environments (Fig. 1.3). Both the coral host and its symbiotic dinoflagellate partners (zooxanthellae) possess enzymes enabling rapid ammonium (NH_4^+) assimilation from the surrounding seawater (Grover *et al.* 2002; Yellowlees *et al.* 2008; Stambler 2011; Pernice *et al.* 2012; Kopp *et al.* 2013). Moreover, the zooxanthellae are also capable of utilizing nitrate (NO_3^-) as a nitrogen source (Grover *et al.* 2003; Kopp *et al.* 2013). Both the animal tissue and the alga assimilate dissolved organic nitrogen (DON) from the surrounding seawater, with preference to urea and dissolved free amino acids (Grover *et al.* 2006,2008; Kopp *et al.* 2013). Finally, coral polyps are also active particle and zooplankton feeders (Ferrier-Pagès *et al.* 2003; Mills *et al.* 2004a).

Nano-scale secondary ion mass spectrometry (NanoSIMS) studies have recently shown that assimilation of both organic and inorganic N sources resulted in rapid incorporation of nitrogen into uric acid crystals, forming temporary N storage sites within the dinoflagellate endosymbionts (Kopp *et al.* 2013). Another study using a similar methodology showed that coral larvae acquire additional nitrogen (in the form of NH_4^+) that has been previously taken up from the environment by bacterial partners (Ceh *et al.* 2013). These results, taken together, draw a picture of corals as opportunistic organisms, which rapidly assimilate and store N from the environment as soon as a source is available.

Other experiments suggest that in coral reef habitats the growth and abundance of zooxanthellae within the coral host is limited by the availability of dissolved inorganic N (Falkowski *et al.* 1993). On the other hand, the presence of N_2 fixers within the host is correlated with higher cell division rate and population size of the endosymbiotic zooxanthellae (Lesser *et al.* 2007; Olson *et al.* 2009). Therefore, in the highly N-depleted waters that characterize most coral reefs, the presence of diazotrophs (Fig. 1.3), thriving in symbiotic association with the corals and their unicellular algae, suggests that N_2 fixation may be an important additional source of N within the host and may enhance primary productivity. In this multi-partner symbiotic system (holobiont) (Knowlton and Rohwer 2003; Krediet *et al.* 2013), the animal host and the zooxanthellae possibly both benefit from the N fixed by the diazotrophs (Fig. 1.3), while both the coral and the diazotrophic bacteria receive the photosyntates (i.e. any product of photosynthesis) produced by the dinoflagellate algae. These speculations, together with the approaches and methodologies which have only recently become available (Fig. 1.4), opens an attractive and feasible area of study to identify metabolic interactions among the partners in cnidarian-dinoflagellate-diazotroph symbioses.

Since symbioses are widespread in coral reef environments and may be found in a variety of benthic reef organisms other than corals, such as in sponges, mollusks and foraminifera (Weisz *et al.* 2010), there is a good chance that benthic N_2-fixing symbionts are widespread as well. However, further research is needed to examine the likely mutual relationship between C and N_2 fixers associated with benthic reef organisms that enable reef communities to overcome N limitation. The quantitative

importance of N_2-fixing symbiotic (internal and/or external) associations in coral reef ecosystems is not debated, and we have only begun to investigate the distribution and diversity of diazotrophic populations associated with benthic reef organisms and substrates. Furthermore, the role of diazotrophs in contributing to primary production and growth and their potential susceptibility to climate change still needs to be resolved. As mutualisms (and nutritional mutualisms in particular) bind different species to a common fate, their breakdown as a result of climate change may enhance biodiversity loss and ecosystem disruption (Kiers *et al.* 2010). It is therefore of the highest priority to investigate the role of N_2-fixing symbioses in coral reef environments.

Human-induced climate change and potential effects on N_2 fixation

Global warming

There is now a strong body of evidence documenting that Earth's climate is changing, and that these changes are largely ascribable to human activities (IPCC 2007). In the context of climate change, one of the major consequences affecting the oceans is global warming, which is particularly exacerbated by increasing concentrations of greenhouse gases (mainly carbon dioxide, CO_2) produced by burning of fossil fuels and deforestation (IPCC 2007). Recent research stresses once more that rigorous and rapid policy decisions are needed in order to save most tropical coral reefs (Frieler *et al.* 2013) because of their high susceptibility to ocean warming, the latter often resulting in coral bleaching (i.e., the loss of their photosynthetic dinoflagellate endosymbionts) and subsequent mass mortality (Hoegh-Guldberg 1999).

The process of N_2 fixation is not intrinsically limited by temperature, and active diazotrophs have been found operating at near freezing temperatures (Bordeleau and Prévost 1994) and at hydrothermal vent fluids (92°C) (Mehta and Baross 2006). However, heterocystous cyanobacteria (i.e., cyanobacteria with specialized cells – heterocysts – protecting nitrogenase from O_2 inhibition) are rare in warm tropical oceans. It has been suggested that the reduced gas solubility and increased respiration rates in warmer waters make the possession of heterocysts under such conditions disadvantageous (Stal 2009), favouring non-heterocystous forms. Moreover, culture studies with the free-living non-heterocystous cyanobacterium *Trichodesmium* spp. showed enhancement of N_2 fixation and growth under warmer temperatures (Hutchins *et al.* 2007; Levitan *et al.* 2010a). Similar diazotrophs (e.g., other *Oscillatoria*) are widespread in benthic environments, so that increases in N_2 fixation are likely to appear here as well. Nevertheless, heterocystous cyanobacteria form mats in warm coral reef environments (e.g. *Anabaena*)(Charpy *et al.* 2012a). Therefore, although high temperatures may represent a physiological constrain to the geographic distribution of certain heterocystous species, others may thrive in the range of temperatures expected for future oceans. Thus, much research is needed on the effects of increased temperatures on benthic diazotrophs and their physiology and activity (Fig. 1.4) if we want to understand the consequences of global warming on the reef N cycle.

Indeed, rising average sea surface temperatures (SST) resulting from global climate change have the potential to increase the amount of N fixed globally (Karl *et al.* 2002; Rijkenberg *et al.* 2011), and particularly in coral reef environments (Paul *et al.* 2005), due to both increased physiological rates of N_2 fixation as well as increasing N_2 fixing cyanobacterial populations (Paerl and Huisman 2008). Global warming may in fact further exacerbate phase shifts from corals to algae in reefs subjected to coral bleaching and related coral mortality providing more space for turf, macroalgae and filamentous cyanobacteria and less space for coral recruitment (Kuffner and Paul 2004; Kuffner *et al.* 2006; Hughes *et al.* 2007). As a result, this may increase suitable substrate for diazotrophy (Davey *et al.* 2008), particularly since the frequency and extent of mass bleaching events are predicted to increase. Indeed, skeletons of thermally bleached corals showed rates up to 30 times greater than those measured on live corals (Davey *et al.* 2008), highlighting the potential for a substantial change in N inputs in reef ecosystems which suffered large scale coral death.

Global warming could also lead to an escalation of harmful cyanobacterial blooms (Paerl and Huisman 2008) of species such as the toxin producing genus *Lyngbya* (Albert *et al.* 2005; Paul *et al.* 2005), as well as species that have been linked with black band disease (BBD) in corals, including *Phormidium* and others (Rosenberg and Loya 2004), some of which are capable of fixing N_2. In the tropics, periods with high SST have been increasing in both frequency and extent worldwide in the past 20 years, consequently increasing the necessity for research aiming to understand the effects of such events on the benthic diazotrophic communities inhabiting coral reefs (Fig. 1.4), particularly after algal blooms or bleaching events which may provide substrate and conditions for diazotrophy.

Finally, the process of N_2 fixation by symbiotic diazotrophs associated to reef primary producers and its contribution to C fixation will likely be affected, as the balance between symbiont and host interaction is very sensitive to environmental conditions (Knowlton 2001; Krediet *et al.* 2013). As internal or external symbionts of specific coral reef organisms, N_2-fixers may be particularly important for providing nutrients to the host during stressful conditions, such as temperature-induced coral bleaching events, when other symbionts (e.g., zooxanthellae) are lost (Fine and Loya 2002; Vega Thurber *et al.* 2012). Future research looking at the metabolic interactions in the coral holobiont might therefore incorporate measurements of nitrogenase activity (and gene expression) under different temperatures and bleaching conditions. The latter results could help by revealing whether diazotrophs play a role in the metabolism of the coral holobiont, giving unprecedented insights into their functions in a changing ocean.

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 and 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.

Coral reef ecosystems are highly dynamic costal systems naturally subject to a high degree of climatological, physical, and biogeochemical variability resulting in diel and seasonal fluctuations in CO_2 partial pressure (pCO_2) and seawater *pH* (Hofmann *et al.* 2011; Massaro *et al.* 2012). However, the steady increase in atmospheric CO_2 is already shifting the baseline of seawater *pH* in coral reef habitats towards values at which decreases in calcification can cause entire reef systems to fall below the balance between calcification and erosion (Hoegh-Guldberg 2011). To some degree, reef ecosystems are predicted to react and adapt to these changes in seawater carbonate chemistry, and recent evidence suggest that these adaptations may partially offset the expected changes in seawater *pH* (Andersson *et al.* 2014). However, it is paramount to understand effects of OA on reef organisms and ecosystems before losing their biodiversity and functioning, especially since "pristine" reefs are essentially already gone (Knowlton and Jackson 2008)

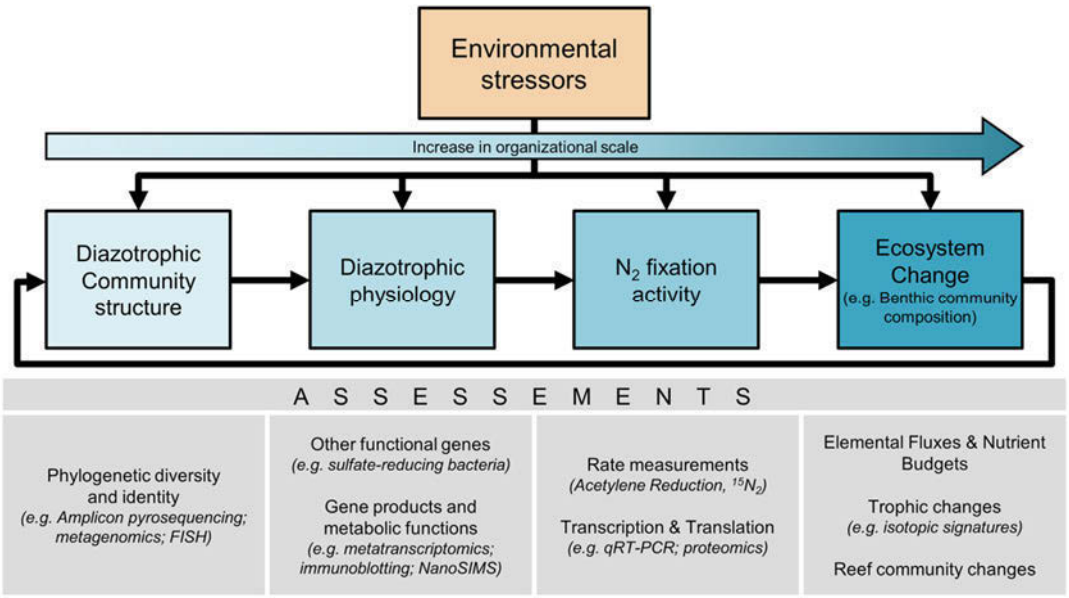

Figure 1.4. Conceptual diagram showing the structural and functional characterization techniques and approaches useful for assessing environmentally induced changes of the diazotrophic community along various organizational scales, ranging from genome to reef scale.

A wide range of reef organisms have been studied under the pH/pCO_2 conditions expected to occur for the end of the century, and the responses are variable. OA effects vary from species to species (Fabricius *et al.* 2011), but are collectively anticipated to be negative on coral reef ecosystem engineers (Wild *et al.* 2011), with CO_2 concentrations above 1000 *ppmv* (*parts per million* by *volume*) resulting in bleaching and productivity loss (Anthony *et al.* 2008). On the other hand, fleshy non calcifying algae and sea grasses flourish under OA conditions (Fabricius *et al.* 2011; Porzio *et al.* 2011) as the additional CO_2 acts as substrate for photosynthesis. Moreover, the decline in grazers and in calcifying epiphytes increases algae development (Hall-Spencer *et al.* 2008). These results suggest that coral reef benthic community composition may adapt and change in response to the increase in acidity towards communities dominated by primary producers other than corals. This would determine drastic

changes in ecosystem functioning with strong feedback on all reef biogeochemical cycles and specifically the N cycle and N_2 fixation (Fig. 1.4). In this context, future OA research should focus on the effects of changes in benthic community structure on the associated diazotrophs, their activity and overall contribution to new N on the reef. Studies addressing this issue may exploit natural pH gradients such as the one in Papua New Guinea (Fabricius *et al.* 2011), which can be used as a natural model to study ecosystem-level effects of OA on N_2 fixation in future coral reef habitats.

However, OA may also impact the microbial community and their physiology directly. Microorganisms comprise the largest diversity and biomass of all marine biota, yet how they may be affected by ocean acidification (OA) remains uncertain (Joint *et al.* 2011). Recent findings emphasize their high sensitivity to expected near-future *pH* changes and highlight the importance of assessing implications of microbial shifts for host health and coral reef processes (Webster *et al.* 2012). First results looking at the coral-associated microbes also suggest a drastic change in microbial composition in the coral mucus, tissue, and skeleton under OA conditions (Meron *et al.* 2011). The physiological effects of increasing pCO_2 on N_2 fixation have very recently been realized, but research up to now only focused on planktonic diazotrophs and cultured isolates. N_2 fixation by the filamentous non-heterocystous cyanobacterium *Trichodesmium* spp. responded positively to increased pCO_2 (Barcelos e Ramos *et al.* 2007). High pCO_2 levels strongly enhance *Trichodesmium* N_2 and C fixation rates, along with its filament length and biomass (Hutchins *et al.* 2007; Levitan *et al.* 2007; Kranz *et al.* 2009; Lomas *et al.* 2012). However, recent findings 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.* 2010b), 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. In heterocystous species such as *Nodularia spumigena* rising pCO_2 had an overall stimulating effect on C and N_2 fixation, as well as on cell growth (Wannicke *et al.* 2012), whereas ocean acidification had no effects on N_2 fixation rates 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. Low dissolved iron concentrations may also limit the response to higher pCO_2, as the availability of iron influences N_2 fixation by affecting the synthesis of the Fe-rich proteins of nitrogenase enzyme complex (Kustka *et al.* 2002; Fu *et al.* 2008; Shi *et al.* 2012). Since the bioavailability of dissolved Fe is expected to decline because of ocean acidification (Shi *et al.* 2010), the increase in global oceanic N_2 and C fixation due to anthropogenic CO_2 enrichment may be tempered (Fu *et al.* 2008).

However, it seems clear that OA also has the potential to substantially alter benthic N_2 fixation, indicated by the finding that *Trichodesmium* N_2 and C fixation response to elevated pCO_2 was the most pronounced physiological response yet reported for marine microbes (Hutchins *et al.* 2009; Liu *et al.* 2010). Similar diazotrophs (i.e., filamentous types) to those found in the open ocean also occur in the benthic environment, particularly in coral reefs, where they may be fundamental in sustaining

the high gross primary productivity. Moreover, cyanobacteria are also responsible for deposition of carbonate structures in tropical environments (Steppe *et al.* 2001), and their study might give precious insight into the effects of OA on benthic diazotrophy. For example, perturbation experiments may be performed looking at the effect of increased pCO_2 on C and N_2 fixation in these microbial mats. These studies should also be expanded to other relevant benthic diazotrophs commonly inhabiting coral reefs. These investigations would help in understanding the consequences of increasing anthropogenic CO_2 on the process of N_2 fixation in coral reef ecosystems (Fig. 1.4).

Ocean eutrophication

During the past century, humans have significantly altered the balance between new N inputs and N losses in the marine environment through the extensive use of synthetic N fertilizers in agriculture, fossil fuel combustion and coastal urbanization (Codispoti *et al.* 2001; Schlesinger 2009). Over this time frame, terrigenous discharge and atmospheric N emissions have increased 10-fold and continue to grow as human development expands in coastal watersheds (Howarth *et al.* 1996). This in turn has the potential to affect N_2 fixation activity and community composition of diazotrophs in the marine environment.

The high energetic costs associated with N_2 fixation have resulted in the idea that this process will be suppressed as soon as N compounds are sufficiently available in the surrounding water. However, experimental work demonstrated that N_2 fixation still occurs at high concentrations of ambient nitrate (Mulholland *et al.* 2001; Voss *et al.* 2004; Holl and Montoya 2005; Moisander *et al.* 2010; Sohm *et al.* 2011a; Sohm *et al.* 2011b; Großkopf and LaRoche 2012), and recent work detected high N_2 fixation rates associated with sediments from an eutrophic estuary affected by groundwater discharge (Rao and Charette 2012). This may be attributed to enhanced heterotrophic N_2 fixation due to organic substrate availability promoting oxygen (O_2) consumption and protecting the enzyme nitrogenase from inactivation (Rao and Charette 2012).

Several studies revealed that nutrients other than N such as dissolved inorganic phosphate (DIP) and dissolved organic matter (DOM) as well as trace metals (e.g. Fe, Mo), are mostly limiting N_2 fixation in the open ocean (Wu *et al.* 2000; Kustka *et al.* 2002; Mills *et al.* 2004b; Arrigo 2005; Moutin *et al.* 2005) 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). Moreover, net primary production in shallow tropical carbonate systems such as coral reefs is often P limited rather than N limited (Smith 1984), but may become N limited as anthropogenic nutrient enrichment accelerates and rates of sediment P adsorption decrease (Howarth *et al.* 1995; Howarth and Marino 2006). This change might allow diazotrophic microorganisms to proliferate, rather than suppress N_2 fixation.

Indeed, one of the major threats associated with marine eutrophication is the proliferation and expansion of cyanobacterial harmful algal blooms (Paerl 1997; Paerl and Huisman 2009). Since cyanobacteria have a high flexibility in exploiting various N sources (through N_2 fixation and uptake of organic or inorganic N compounds), their ability to fix N_2 is advantageous over non-N_2 fixing phytoplankton species, especially under N limiting conditions (O'Neil *et al.* 2012). Marine cyanobacterial blooms (e.g. *Lyngbya*, *Trichodesmium* or *Synechococcus*) can significantly alter the competition within the phytoplankton community, thereby further threatening the stability and functioning of the whole ecosystem (O'Neil *et al.* 2012). Furthermore, the high algal biomass during blooms can decrease benthic light availability, while its subsequent microbial decomposition significantly reduces oxygen availability near the water-sediment interface. Oxygen depletion in bottom waters affects nutrient cycling within the hypoxic zone as less nutrients are retained by the sediment and high amounts of phosphorus and trace metals (iron and molybdenum) are released to the water column (O'Neil *et al.* 2012). This can further stimulate the growth of planktonic N_2 fixing cyanobacteria thereby enhancing the effects of eutrophication (Conley *et al.* 2009). At the same time, the role of heterotrophic N_2 fixing bacteria is not well understood and needs to be elucidated. These bacteria can fix N_2 in organic-rich, anoxic sediments even in the presence of large amounts of ammonium (Howarth *et al.* 1988) and in the case of SRBs their N_2 fixation rates were positively correlated with plankton bloom activities (Bertics *et al.* 2013).

In coral reef oligotrophic environments, characterized by high light levels reaching the bottom, symbiotic or epiphytic cyanobacteria associated with benthic organisms (e.g. seagrasses, corals) often fix N_2 at high rates and may significantly contribute to the N inputs (Carpenter and Capone 2008). 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)). Nevertheless, in the ENCORE (The Effect of Nutrient Enrichment on Coral Reefs) program (Koop *et al.* 2001), 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. Indeed, Koop and colleagues (2001) 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 the 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). Another study investigated the effects of a mass coral spawning event on the N cycle in carbonate reef sediments and found a rapid increase in benthic production when more N became available, reflecting strong short-term N limitation (Eyre *et al.* 2008). However, coral reef ecosystems may ultimately be P limited because N can be replenished via N_2 fixation in the longer term (Eyre *et al.* 2008). As coral reefs are highly susceptible to nutrient inputs due to their proximity to coastal areas (Pastorok and Bilyard 1985) and the balance of symbiont-host interactions can be very sensitive to changing environmental conditions, changes in N_2 fixation activity following eutrophication will affect N cycles on both the organism and ecosystem level (Fig. 1.4). Therefore, these hypotheses deserve to be investigated by future research, which should focus on the effects of anthropogenic nutrient inputs

on N_2 fixation by diazotrophs associated with benthic reef organisms and the coral reef ecosystem. Manipulative experiments may help disentangle the effects of the different forms of inorganic and organic P and N on these microbes, on their physiology and activity and therefore on the potential effects on coral reef nutrient cycles.

Ocean deoxygenation

A major consequence of global climate change, which has only recently received consideration is the decrease in the dissolved O_2 content in the World's oceans (Keeling *et al.* 2010). This phenomenon, called "deoxygenation", is produced as a result of both the decrease in solubility of O_2 and the increased upper ocean stratification due to global warming, the latter reducing in turn the O_2 supply to the ocean interior (Sarmiento *et al.* 1998; Keeling and Garcia 2002). Additional O_2 loss caused by eutrophication-induced stimulation of microbial respiration is likely to worsen the problem (Breitburg *et al.* 2009; Conley *et al.* 2009).

Coral reefs thrive in shallow well-mixed oxygenated waters and are dominated by photosynthetic organisms. These ecosystems are therefore generally believed to be exempt from the effects of deoxygenation. However, episodic events have been recorded during which dissolved oxygen (DO) levels dropped to hypoxic conditions as a consequence of coral spawning events (Simpson *et al.* 1993) or phytoplankton blooms (Guzmán *et al.* 1990), eventually causing extensive mortality of corals and other reef animals over wide areas. Moreover, coral reef benthos naturally experiences strong shifts in DO concentrations on a diel basis as the community shifts from net photosynthesis during the day to respiration during the night. A study from a coral reef platform on the Great Barrier Reef showed that DO can range from 2.1 mg O_2/L after midnight to more than 10.8 mg O_2/L (the limit of the instrument) in the early afternoon (Kinsey and Kinsey 1967). Other studies looked at DO levels in the diffusive boundary layer surrounding stony corals, and found extreme diel fluctuations, with concentrations varying from supersaturation during the day to anoxia at night (Shashar *et al.* 1993; Kühl *et al.* 1995). A later study demonstrated that the presence of sleep-swimming fishes inside coral heads may indeed be considered a mutualism where the fishes find refuge from predation while mitigating hypoxia in the coral branches (Goldshmid *et al.* 2004). Finally, hypoxic zones occur at the competing interface between algae and corals (Smith *et al.* 2006; Barott *et al.* 2009; Barott *et al.* 2012; Wangpraseurt *et al.* 2012). Thus, hypoxia has been suggested to play a significant role in coral tissue mortality during coral-algae interaction processes (Haas *et al.* 2014). As human impacts cause a global decrease in oxygen availability in the water column, eutrophication increases and algae become more abundant on reefs, temporally and spatially restricted hypoxic conditions in coral reefs may become more common. Therefore, research would benefit from studies looking at the alterations of microbial-driven biogeochemical processes occurring in hypoxic zones of coral reefs, which are likely to further expand in the future. In this context, studying diazotrophs and their physiology (Fig. 1.4) in coral reef hypoxic microenvironments such as coral-algae interactions or looking at pulse ecosystem-level

hypoxic conditions on the input of fixed N on the reef may give insights into the effects of decreased oxygen availability on marine diazotrophy.

Generally, low O_2 levels favour nitrogenase activity as this is irreversibly inhibited by molecular O_2 (Berman-Frank *et al.* 2003). Many diazotrophs are only active under anaerobic conditions, others respire to draw down O_2 levels, or bind O_2 with proteins such as leghemoglobin (Wittenberg *et al.* 1974; Robson and Postgate 1980). Under anoxic conditions (no O_2 present) the microbial community tends to be dominated by SRBs, particularly when nitrate is exhausted (Keeling *et al.* 2010). Among the microorganisms in the benthos, SRBs can fix N_2 in a variety of benthic habitats (Nielsen *et al.* 2001; Steppe and Paerl 2002; Bertics *et al.* 2010; Bertics *et al.* 2012) and may facilitate N_2 fixation in sediments beneath hypoxic waters (Bertics *et al.* 2013). SRBs are important members of the diazotrophic community in seagrass rhizosphere sediments (Capone 1982; McGlathery *et al.* 1998) as well as in sediments colonized by macroalgae of the genus *Caulerpa* (Chisholm and Moulin 2003).

In summary, these results imply a significant effect of decreasing O_2 concentrations on the process of N_2 fixation. Future research efforts should concentrate on the activity of SRBs in coral reef habitats and on their potential role in hypoxic sediments and coral-algae interactions. As O_2 supply is decreasing in warmer climates, and coastal hypoxia is increasing in the global coastal zone, where it is recognized as a major threat to biota (Steckbauer *et al.* 2011), major changes are also likely to occur in diazotrophs associated with coral reefs, particularly since several reefs are subjected to a steady coastal influence.

Ultraviolet radiation (UVR) stress

Anthropogenic inputs of chlorinated fluorocarbons and the consequent decrease of stratospheric ozone have already led to an increase in the amount of harmful ultraviolet radiation (UVR) reaching the biosphere (Stolarski *et al.* 1992; Kerr and McElroy 1993; Madronich *et al.* 1998). Furthermore, increasing CO_2 atmospheric concentrations and resulting climate change will deeply alter the tropospheric ozone budget and increase the ultraviolet index, which would have further consequences for the health and functioning of marine ecosystems (Hegglin and Shepherd 2009).

UVR can penetrate to significant water depths in marine and aquatic ecosystems and may determine significant biological effects on marine biota (Tedetti and Sempéré 2006; Lesser 2008). Moreover, the low solar zenith angle and the natural thinness of the ozone layer over tropical latitudes together with the high transparency of the water column result in the high UVR irradiances that marine organisms experience in shallow-water tropical coral reef environments (Shick *et al.* 1996; Banaszak and Lesser 2009).

A large body of evidence is available demonstrating the direct and indirect effects of UVR (e.g. DNA damage, photooxidative stress, bleaching, detrimental effects on reproduction and on larval development) on corals and other reef associated biota (reviewed in (Banaszak and Lesser 2009). However, studies looking at the effects of UVR on the process of N_2 fixation only focused on

planktonic cyanobacteria. The consequences of increasing UVR reaching the benthos in coral reefs and their associated N_2 fixers is still to be investigated. Therefore, future research needs to give more attention to the consequences of UVR on benthic reef diazotrophs, particularly since even small anthropogenic increases in UVB levels will have sublethal physiological manifestations in coral reef macroorganisms (Shick *et al.* 1996).

Ultraviolet radiation, both UVA (320–400 nm) and UVB (290–320 nm), can alter photosynthesis and growth in cyanobacteria (Vincent and Roy 1993). However, cyanobacteria have developed several defense mechanisms helping them to successfully grow and survive in several habitats receiving high solar UVR (Singh *et al.* 2010) such as coral reefs. N_2 fixation is also suppressed either directly or indirectly by UVB radiation (Singh *et al.* 2010) due to the extreme sensitivity of the nitrogenase enzyme (Tyagi *et al.* 1992; Kumar *et al.* 2003; Lesser 2008). A 57 % decline in N_2 fixation occurred in cultures of *Anabaena* sp. exposed to UVR, despite an increase in both the concentration of UV photoprotectants and the activity of antioxidant enzymes (Lesser 2008), while several rice-field cyanobacteria showed complete loss of nitrogenase activity (Kumar *et al.* 2003). These results show that N_2-fixing cyanobacteria are particularly affected by UVR (Lesser 2008). UVR effects on this group of prokaryotes or on other N_2-fixing microorganisms may therefore deeply affect the input of new N and the biogeochemical cycling of this essential macronutrient in the oceans. However, UVR may also determine indirect effects on the process of N_2 fixation in coral reefs. For example, UVR can cause mass coral bleaching and following coral mortality across wide reef areas (Drollet *et al.* 1995). These mortality events, in turn, provide free space on dead coral colonies, which is often colonized by turf algae and cyanobacterial mats (Davey *et al.* 2008). Changes in the benthic community composition of coral reefs will consequently have cascading effects on the benthos-associated microbes and their activity (Fig. 1.4), therefore potentially altering the inputs of new N at the ecosystem scale. As these ecosystems are already subjected to high UV irradiances, which are expected to further increase in the future, the effects of UVR on benthic reef N_2 fixation deserve attention.

Conclusions and perspectives

The functioning of coral reefs is strongly connected with the maintenance of the oligotrophic conditions in which these ecosystems thrive. Variations in the inputs of nutrients to the reef will perturb the tightly coupled recycling and biogeochemical cycles of reef ecosystems, with consequences that are far from being understood. Recent evidence demonstrated that increases in dissolved inorganic nitrogen concentrations decrease the thermal tolerance of corals and increase their susceptibility to bleaching (Wooldridge 2009; Wiedenmann *et al.* 2013; Vega Thurber *et al.* 2014). On the other hand, turf and macroalgae are favoured under high nutrient availabilities (Jessen *et al.* 2013). Therefore, these changes may eventually result in phase shifts from coral to algae-dominated communities. It is therefore of the highest priority to investigate and understand the relevance of N_2

fixation to the whole reef N cycle, in order to predict the effects of human interference in coral reef ecosystems.

Although associations with N_2-fixing microbes occur in several benthic reef organisms, we currently lack a full understanding of the benefits and costs in many of these associations. There are no studies investigating the abundance and distribution of mutualistic interactions between C and N_2 fixers in reef organisms, which may significantly contribute to the overall C and N_2 fixation within the coral reef. As primary productivity is mostly N-limited, this additional source of N from a symbiotic partner represents a further adaptation of these organisms to flourish in oligotrophic reef waters. Gaps of knowledge are present in how N_2 fixation by benthic diazotrophic reef associations will respond to global climate change and to the increasing anthropogenic CO_2 dissolving into the oceans. Interacting and synergistic effects of global stressors with local disturbances, such as industrial pollution, sewage and land run off, dredging, overfishing and destructive fishing, have scarcely been studied (Harnik *et al.* 2012; Ateweberhan *et al.* 2013) but will be essential to understanding and predicting coral reef biogeochemical cycles under conditions of global change.

This paper highlights the paramount role of N_2 fixation in coral reef ecosystems, and the vast scope of global environmental impacts that are predicted to affect diazotrophy in future reef habitats. As the reef paradox still needs to be resolved, N_2 fixation in coral reefs is a topic of the highest priority if we want to understand their functioning before the impact of human alterations will deprive us of a baseline for pristine ecosystems (Knowlton and Jackson 2008). In order to understand potential effects within a reasonable timeline, we suggest several high priority topics: 1) to identify ecological distribution patterns of important benthic diazotrophs; 2) to expand the focus to internal and external associations of N_2-fixers with benthic eukaryotic organisms; 3) to investigate the potential effects of global and local environmental stressors on the metabolic activity and stability of these partnerships; 4) to scale the effects of environmental stressors on the input of fixed N to the entire reef ecosystem. Moreover, careful site selection should be considered, where we can utilize natural habitats which currently undergo similar climate driven stressor(s) expected for the future, i.e. CO_2 seeps.

Given the difficulty in isolation and cultivation of microorganisms, including symbiotically associated types, several culture independent approaches (Fig. 1.4) are useful for investigating diazotrophic community structure and metabolic functions in coral reef habitats. Finally, if we want to scale the consequences of changing microbial communities to the effects on the reef ecosystem level, it is essential that we use a multidisciplinary approach. Bridging the gaps among molecular ecology, biochemistry, physiology and coral reef ecology will be important, as physiological measurements of N_2 fixation rates as well as ecological and biogeochemical approaches are necessary for understanding the functioning of such a complex ecosystem.

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Chapter 2
The study site: visual evidence of structural habitat loss and community shift

Abstract

This study compares the same reef section (25 m^2) analysed almost four decades ago by Professor Helmut Schuhmacher and provides visual evidence of benthic community changes over this span of time at the study site. The key results of this chapter have been produced using raster graphics editor software and a tool for the determination of percentage benthic cover using photographs (CPCe - Coral Point Count with Excel extensions). The short text that follows wants to support the information visible to the naked eye with the main data obtained from the analysis of the pictures.

This chapter is intended for publication as 'reef site' in *Coral Reefs*: **Cardini U**, van Hoytema N, Al-Rshaidat MMD, Schuhmacher H, Wild C, Naumann MS. Structural habitat loss and community shift recorded after 37 years on a Red Sea fringing reef (1976 - 2013).

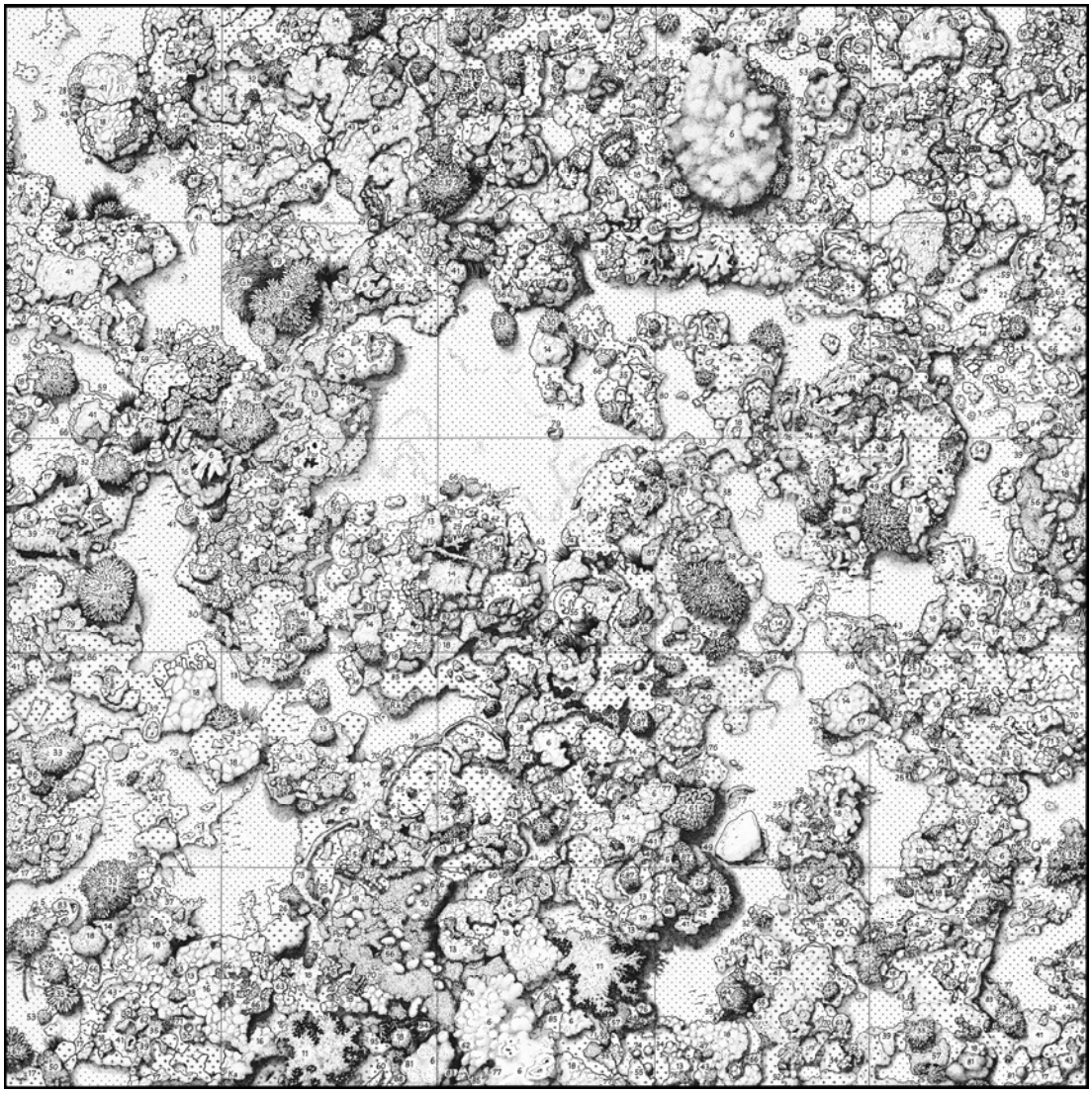

Figure 2.1. Benthic reef community in the permanent quadrat as recorded in January 1976.

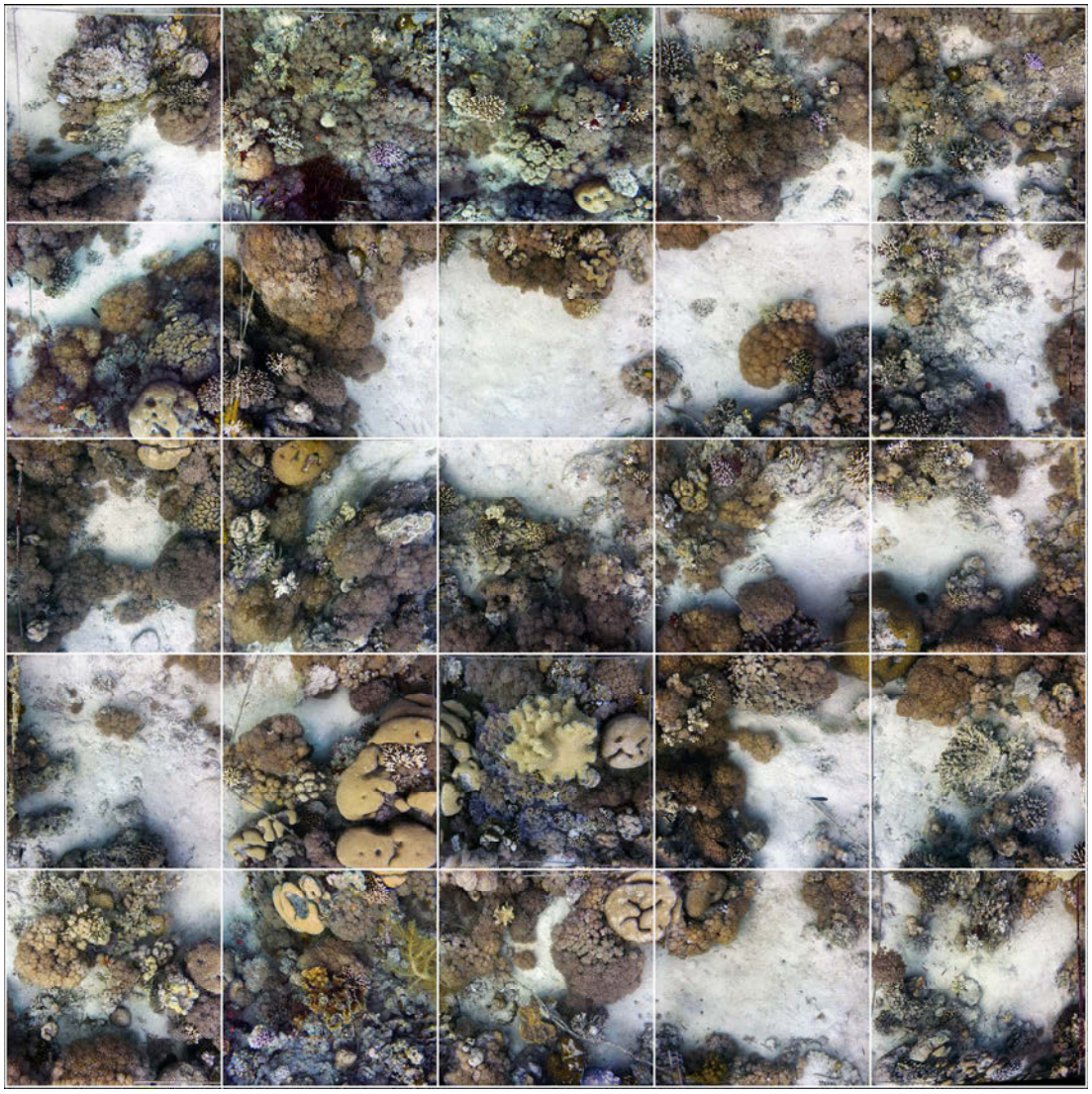

Figure 2.2. Benthic reef community in the permanent quadrat as recorded in January 2013.

Main text

Biodiversity and species abundance in coral reef ecosystems are sustained by structural complexity of the reef carbonate framework (Graham and Nash 2013). Recent carbonate budgets and topographic complexity models reveal reduced net reef accretion with concomitant loss of functional habitat complexity affected by global and local anthropogenic stressors (Kennedy *et al.* 2013). However, visual evidence for change in benthic reef communities and structural habitat loss derived from permanent long-term monitoring sites is scarce. The benthic reef community composition of the permanent reef quadrat U-7 (size: 5x5 m) established in January 1976 (Fig. 2.1) at 10 m water depth on the fore reef of a Red Sea fringing reef south of Aqaba (Gulf of Aqaba, Jordan) was reassessed after nearly four decades in January 2013 (Fig. 2.2, Appendix II, Mergner and Schuhmacher 1981). High resolution sectional photographs were combined to provide a comparative overview of the entire test area (Fig. 2.2). Image analysis revealed drastic changes in benthic reef community composition by a relay in dominance of hermatypic (Scleractinia, *Millepora*) to ahermatypic (mostly Alcyonaria) cnidarians, with hermatypic cover declining from 22 to 15% and ahermatypic cover rising from 21 to 33% between 1976 and 2013. At once, a visible increase in sand area (26 to 31%) and substantial decreases in dead coral skeleton and rock cover (30 to 12%) testify major structural habitat loss with implications for future net reef accretion and associated biodiversity. Global (i.e., ocean acidification and warming) as well as local (i.e., coastal development, overfishing) anthropogenic stressors may qualify responsible for this ecosystem degradation.

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Part II

Dinitrogen and Carbon fixation by coral reef waters and benthic organisms

Chapter 3
Water column: the desert surrounding coral reefs

Abstract

The northern Red Sea, because of its high-latitude location, experiences strong seasonality in environmental conditions. This allows the study of regulatory effects by key status parameters (temperature, inorganic nutrient and organic matter concentrations) on process parameters (primary production and dinitrogen (N_2) fixation) and planktonic community composition in the water column above coral reefs. Knowledge on interactions between these parameters is lacking. Therefore, this study, for the first time in high latitude coral reef waters, measured status and process parameters, and picoplankton community character using a comparative approach between mixed (January – April) and stratified (September – November) water column scenarios in 2013. Findings revealed that inorganic nutrient concentrations were significantly higher in the mixed compared to the stratified season. Concurrently, daily gross primary production decreased 4-fold from the mixed to stratified season, while N_2 fixation did not change significantly. The phytoplanktonic community changed from dominance by picoeukaryotes to that by *Prochlorococcus* sp. and indications were found for a diazotrophic community shift from its autotrophic to its heterotrophic component. Primary production was primarily regulated by inorganic N concentrations, while dissolved organic carbon concentrations affected both primary production and N_2 fixation, emphasizing the importance of the microbial loop in planktonic tropho-dynamics of Red Sea coral reefs. N_2 fixation could potentially contribute 3.4 % of N needed for primary production in the mixed season. However, this contribution increased drastically to 20.8 % in the stratified season, indicating planktonic N_2 fixation as an important potential source of N to phytoplankton during very oligotrophic summer conditions.

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Introduction

The Gulf of Aqaba forms one of the northern tips of the Red Sea. Its desert coasts are fronted by fringing coral reefs that experience relatively strong variation in light availability and water temperature for warm water coral reefs due to their high latitude location. The annual fluctuation in sea surface temperature (21 – 29 °C) combined with relatively warm deeper water layers (year round ~ 21 °C, water depth > 200 m) result in an annual cycle of deep water mixing from December until May and stratification down to 200 m water depth from June until November (Carlson *et al.* 2014). Inorganic nutrients are brought up to surface water layers during deep water mixing, while they are trapped in deeper waters during stratification, creating extreme oligotrophic conditions in coral reef surrounding surface waters (Rasheed *et al.* 2002; Silverman *et al.* 2007; Rasheed *et al.* 2012). These local conditions offer the rare opportunity to study the effects of seasonal variation in key environmental factors on important processes such as primary production within coral reefs.

In addition to receiving allochthonous energy and nutrients in the form of plankton, and particulate and dissolved matter from offshore, coral reefs thrive in oligotrophic conditions particularly due to autochthonous creation of organic carbon (C) and nitrogen (N) containing nutrients through photosynthetic primary production and prokaryotic dinitrogen (N_2) fixation and efficient internal recycling of those materials within the ecosystem (Hatcher 1997). This recycling can occur through strong benthic-pelagic coupling of dissolved and particulate organic matter and nutrients (Yahel *et al.* 1998, Wild *et al.* 2004; Chipman *et al.* 2012). Benthic-pelagic coupling is mediated by benthic organisms such as corals, algae, and sponges (Haas *et al.* 2010; Naumann *et al.* 2010; de Goeij *et al.* 2013), but also by reef sediment and framework microbiota (Rasheed *et al.* 2002; Wild *et al.* 2009). Planktonic photosynthesis rates in reef-surrounding waters can be 1 to 2 orders of magnitude higher than in oceanic waters offshore (D'Elia and Wiebe 1990; Adey 1998), and N_2 fixation by so-called "diazotrophs" (i.e. prokaryotes capable of N_2 fixation) can facilitate primary production under oligotrophic conditions as found in the Gulf of Aqaba (Capone *et al.* 1997; Karl *et al.* 2002; Furnas *et al.* 2011).

Planktonic primary production in the Gulf of Aqaba and northern Red Sea is dominated by photoautotrophic nano- and picoplankton organisms, where the plankton fraction < 20 µm performs on average 81 % of photosynthesis at 10 m water depth (Al Najjar *et al.* 2007, Qurban *et al.* 2014). Primary production rates range from 0.02 to 3.38 µmol C L^{-1} d^{-1} (Levanon-Spanier *et al.* 1979, Qurban *et al.* 2014), assuming a 12 h period of daylight. The phytoplankton community in the Gulf of Aqaba is characterized by a strong seasonal shift in composition. During the mixed season, eukaryotic algae account for up to 95 % of phytoplankton biomass, while during stratification > 60% of the biomass consists of prokaryotes, in particular *Prochlorococcus* sp. which then may comprise up to 50% of the biomass (Al Najjar *et al.* 2007). The main groups of planktonic diazotrophs in the Gulf of Aqaba are cyanobacteria and proteobacteria, responsible for water column N_2 fixation rates ranging from 0.01 to 1.9 nmol N L^{-1} d^{-1} (Foster *et al.* 2009, Rahav *et al.* 2013). Primary production and N_2

fixation can interact synergistically within or between planktonic organisms: while photosynthesis (and subsequent organic carbon release to the surrounding water) may fuel the energy-demanding process of N_2 fixation, primary production is supported by N_2 fixation providing bio-available N required for proteins needed in photosynthesis (e.g. Foster *et al.* 2011).

Information on planktonic primary production in the Gulf of Aqaba, or even the Red Sea as a whole, is scarce, especially for waters overlying coral reefs (e.g. Levanon-Spanier *et al.* (1979) for the Gulf of Aqaba). In addition, there are only two studies on planktonic N_2 fixation in the Gulf of Aqaba (Foster *et al.* 2009, Rahav *et al.* 2013), and only Rahav *et al.* (2013) provide a first look at conditional interactions between primary production and N_2 fixation. These authors reported indications for a pelagic community shift from autotrophic to heterotrophic diazotrophs from the mixed to the stratified season. To increase our understanding of the regulating factors for planktonic production of organic C and inorganic N-nutrients, which are in high demand by the planktonic community as well as the coral reef below, the objectives of this study were 1) to measure status parameters in a Gulf of Aqaba fringing coral reef over the two distinct seasons of mixing and stratification, 2) to measure abundances of the dominant picoplanktonic groups, 3) to quantify the process parameters primary production and N_2 fixation in the reef-surrounding water over the seasons and 4) to investigate conditional relationships between the measured status and process parameters.

Material and methods

Research area

The fieldwork for this study was conducted at the Marine Science Station (MSS) of the University of Jordan, located 10 km south of Aqaba, Jordan. The MSS is situated adjacent to a marine protected area encompassing a crescent shaped fringing coral reef with a length of ca. 1 km. All water column sampling was performed at 10 m water depth in the fore reef section of this reef (29° 27' 31" N, 34° 58' 26" E). Two extensive fieldwork campaigns were performed in 2013: one during the mixed season (January – April; 12 weeks), and one during the stratified season (September – November; 13 weeks). The studied reef consists of a shallow reef flat surrounded by a carbonate sediment belt at ca. 5 m water depth and a coral dominated middle-fore reef facing the open sea (see Mergner and Schuhmacher (1974) for an extensive description of the studied reef).

Status parameters

Light (lux) and water temperature at 10 m water depth were measured over both campaigns at 1 min frequency with Onset HOBO data loggers (Pendant UA-002-64; temperature accuracy: ± 0.53 °C, spectral detection range: 150 – 1200 nm). The lux measurements were converted to photosynthetically active radiation (PAR) by a conversion factor calculated from a simultaneous minute-by-minute measurement of lux and PAR (08:00 to 14:00 on one day n = 353) using a HOBO pendant logger and

a LI-COR LI192SA underwater quantum sensor: lux = PAR x 52.0, R^2 = 0.83. This value is comparable to the conversion factor given by Valiela (1984): 51.2.

Water samples were collected on a weekly basis during both campaigns for measurement of the status parameters, described in detail below, (n = 4) and primary production (n = 6) in clean high density poly-ethylene (HDPE) containers (volume: 5 L) using SCUBA. All samples were collected between 08:00 and 10:00 within a 10 min time-frame. Sampling was performed 1 m above the seafloor without disturbing the benthos. The samples were transported back to the MSS, and subsamples were taken from the containers within 20 min to measure concentrations of inorganic nutrients (NH_4^+, PO_4^{3-}, and NO_x (NO_x = NO_2^- + NO_3^-)), particulate organic carbon (POC), particulate nitrogen (PN), dissolved organic carbon (DOC), and chlorophyll a (Chl a). Simultaneously, glass incubation chambers (volume: 0.5 L) were filled for measurements of net primary production (Pnet), and biological oxygen demand (BOD) (as described below). Subsamples for inorganic nutrients (50 mL) were filtered through sample pre-rinsed cellulose acetate filters (nominal pore size 0.45 µm), and nutrient concentrations were measured according to Holmes *et al.* (1999), Murphy and Riley (1962), and Strickland and Parsons (1972). NH_4^+ was measured with a Trilogy Fluorometer (Turner Designs), while PO_4^{3-} and NO_x were measured with a JASCO-V630 photometer. Detection limits for NH_4^+, PO_4^{3-}, and NO_x were 0.09, 0.01, and 0.02 µmol L^{-1}, respectively. Subsamples for POC (1L) and PN (2L) were taken from the containers after homogenisation and filtered onto pre-combusted (450 °C, 5 h) GF/F filters (nominal pore size 0.7 µm), which were dried (40 °C for 48 h) and sealed in Eppendorf vials pending analysis. POC and PN filters were placed in silver cups and measured using a EuroVector elemental analyzer (EURO EA 3000; analytical precision ≤ 0.1 % (C) and ≤ 0.03 % (N)) POC filters were acidified (0.1 N HCl) prior to measurement to remove any inorganic carbon. Subsamples for DOC (50 mL) were vacuum filtered (max. pressure 20kPa) through pre-combusted GF/F filters straight into 30 mL HDPE sample bottles. All components of the filtration apparatus and the sample bottles were acid-washed in 0.4 mol L^{-1} HCl for 24 h before being rinsed with MQ water prior to sampling. The first 2 x 10 mL of the DOC samples were used to rinse the sample bottles twice and discarded, after which the remaining 30 mL were filtered and collected. DOC samples were directly acidified with 80 µL of 18.5 % HCl and stored at 4 °C in the dark until analysis by high-temperature catalytic oxidation on a Shimadzu TOC-V_{CPH} total organic C analyser. The analyzer was calibrated with a 10 point calibration using serial dilutions of a potassium hydrogen phthalate certified stock solution (1000 ppm standard, Fluka 76067-500ML-F). Reference water samples (CRM program, Hansell Research Lab, USA, DA Hansell and W Chan; Batch 13, Lot #08-13, 41-45 µmol C L^{-1}), were applied as a positive control after every 10 samples. Each sample was measured five times and analytical precision was < 3 % of the certified value. Chl a subsamples (1L) were filtered onto pre-combusted GF/F filters and stored in the dark at -80 °C. Chl a was later extracted in 90 % acetone for 12 h at 4 °C in the dark and measured on a Trilogy fluorometer using the non-acidification module (CHL NA #046, Turner Designs).

Picoplankton community

Picoplankton samples (2 mL) were collected in March and November for quantification of picoeukaryote, *Prochlorococcus* sp., *Synechococcus* sp., and heterotrophic bacteria abundance. Samples were fixed with 0.1 % paraformaldehyde (final concentration) for 30 min at room temperature, frozen with liquid N, and stored at – 80 °C until analysis. All groups were sorted with flow cytometry (FACSCalibur, Becton Dickinson, 488 nm excitation laser). Picoeukaryotes, Prochlorococcus sp., and Synechococcus sp. were quantified at a flow rate of ~ 0.06 µL min^{-1} for two minutes and gated on an orange versus red fluorescence dot plot using CellQuestPro (BD and Co. New Jersey, USA). Subsamples for heterotrophic bacteria quantification were stained with SYBR Green 1 (conc. 1 per 1000) for 30 min prior to analysis. Heterotrophic bacteria were also sorted at a flowrate of ~0.06 µL min^{-1} but for 1 min and subsequently gated on a side scatter versus green fluorescence density plot. The cytometer flow rate was gravimetrically calibrated according to Current Protocols in Cytometry (Robinson *et al.* 1997).

Process parameters

Primary production

The water samples for measurements of Pnet and BOD were carefully transferred from the sampling containers into 0.5 L glass incubation chambers. Two samples were taken per container, one sample for Pnet and one for BOD measurements. The salinity and temperature corrected dissolved O_2 concentration (mg L^{-1}) was measured with an O_2 optode and a conductivity probe (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany, accuracy: ± 0.5 % of measured value), after which the chambers were directly sealed with gastight glass lids, ensuring no air remained in the chambers. Chambers for BOD measurements were immediately placed in bags made of dense opaque plastic (volume: 10 L) for incubation in the dark. All chambers were incubated in an outside flow-through aquarium (800 L, flow-through 4000 L h^{-1}) fuelled with water pumped from 10 m water depth on the reef to simulate in situ temperatures. PAR in the flow-through aquarium was modified to in situ 10 m water depth levels with layers of plastic mesh following in situ measurements with a LI-COR LI192SA underwater quantum sensor. Pnet incubations were performed from 10:00 until sunset (17:00 - 18:00; depending on the season), while BOD incubations ran for 24 h. At the end of the incubations, chambers were removed from the aquarium, and the O_2 concentration was immediately measured for each chamber under light stirring to ensure a homogenous O_2 concentration.

N_2 fixation

N_2 fixation in the water was measured for seven weeks in the mixed season (four weeks in February and three weeks in April), and seven weeks in the stratified season (three weeks in September, and four weeks in November). N_2 fixation rates were quantified using a modified acetylene (C_2H_2) reduction technique (Capone 1993, Wilson *et al.* 2012). C_2H_2 enriched seawater was produced by bubbling it with C_2H_2 gas freshly produced from calcium carbide. Incubations were performed in 1 L

chambers (n = 8) containing 800 mL fresh seawater taken from the flow-through aquarium described above. Of this 800 mL, 10 % was replaced by C_2H_2 enriched seawater. Chambers were directly sealed with gastight glass lids, after which 10 % of the air headspace was replaced with C_2H_2 gas through a rubber injection port in the lid which was also used for subsequent sampling. Chambers were then incubated under in situ conditions in the flow-through aquarium while being stirred at 600 rpm by Cimarec™ i Telesystem Multipoint Stirrers (Thermo Scientific™) for the duration of the incubation to promote equalizing of gas concentrations between the water and the head space. Control incubations were performed without C_2H_2 addition to measure biological ethylene (C_2H_4) production, as well as with sterile filtered seawater (0.2 µm) to measure inorganic C_2H_4 production from C_2H_2, but no significant C_2H_4 production was found in these control incubations. The incubations lasted for 24 h, and 1 mL gas samples were extracted from a port in the lid at 0 h, 4 h, and 24 h with a gastight syringe. These samples were injected into gas-tight 2 mL vials, previously filled with distilled water, by displacing 1 mL of the water. Vials were frozen upside down pending analysis. Prior to measurements, vials were thawed completely, after which a gas subsample was injected into a reducing compound photometer (RCP) (Peak Laboratories) with a detection limit of 100 ppb for measurement of C_2H_4. The RCP was calibrated with a serial dilution of a C_2H_4 standard (200 ± 4 ppm; Restek, Bellefonte, PA, USA).

Data treatment

PAR values measured per minute were summed for each weekly water sampling day: values in µmol quanta m^{-2} s^{-1} were multiplied x 60 for the minute following each measurement. These 60 sec measurements were then summed for the entire day resulting in mol quanta m^{-2} d^{-1}. Water temperature measured per minute on the water sampling day was averaged over the 24 h period. The O_2 concentration changes over the Pnet and BOD incubation periods were calculated by subtracting start O_2 concentrations from end O_2 concentrations. This concentration difference was corrected for incubation duration, chamber volume, and recalculated to molar equivalents resulting in O_2 fluxes in µmol O_2 L^{-1} h^{-1}. BOD is presented here as a positive flux. Estimates of gross primary production (Pgross) were derived from addition of every BOD measurement to its corresponding Pnet measurement (Pgross = Pnet + BOD). C_2H_4 measurements were recalculated to nmol C_2H_4 in the whole chamber water volume. The C_2H_2 concentration was allowed to equilibrate between headspace and incubation water in the first 4 h, after which C_2H_4 production over 4 h to 24 h were used for flux calculations. Changes in C_2H_4 concentration over time were corrected for incubation duration and volume of water in the chamber, resulting in fluxes of nmol C_2H_4 L^{-1} h^{-1}.

Statistical analyses

The results of replicate measurements for all status and process parameters for each week were averaged prior to statistical analyses. All statistics were performed in SPSS 14.0 for Windows (SPSS Inc. Chicago, USA). Status and process parameters, as well as picoplankton abundances in the two seasons were tested for normality with the Shapiro-Wilk test. Seasonal comparisons were performed

with independent samples T-tests if data were normally distributed and with Mann-Whitney U tests if data lacked normality. Differences were deemed significant at $p < 0.05$. To investigate correlations between all status and process parameters, Spearman Rank correlation tests were performed on the dataset as a whole and on each season separately.

Results

Status parameters

Table 3.1. Status and process parameters in the mixed and stratified seasons. Values are given as mean ± SD. Differences were tested with independent sample t-tests, except for parameters with *, these were tested with Mann-Whitney U tests due to lack of normality. Bold p-values indicate $p < 0.05$. Chl a = chlorophyll a, DOC = dissolved organic carbon, POC = particulate organic carbon, PN = particulate nitrogen, $NO_x = NO_2^- + NO_3^-$, $DIN = NH_4^+ + NO_x$, Pnet = net primary production, BOD = biological oxygen demand, Pgross = gross primary production = Pnet + BOD.

Parameters	Mixed	Stratified	P
Temperature (°C)*	22.34 ± 0.24	25.84 ± 1.00	**<0.001**
Irradiance (mol m^{-2} d^{-1})	4.71 ± 1.27	5.39 ± 1.58	0.264
Chl a µg L^{-1}	0.20 ± 0.03	0.14 ± 0.05	**0.002**
DOC µmol L^{-1}	75.51 ± 6.96	87.36 ± 4.20	**<0.001**
POC µmol L^{-1}	7.90 ± 2.98	7.74 ± 1.79	0.865
PN µmol L^{-1}*	1.08 ± 0.27	0.88 ± 0.17	**0.022**
NH_4^+ µmol L^{-1}	0.52 ± 0.19	0.25 ± 0.12	**<0.001**
PO_4^{3-} µmol L^{-1}	0.11 ± 0.02	0.04 ± 0.02	**<0.001**
NO_x µmol L^{-1}	0.63 ± 0.29	0.19 ± 0.12	**<0.001**
$DIN:PO_4^{3-}$	10.42 ± 2.32	16.52 ± 11.31	0.122
POC:PN	7.16 ± 1.34	8.77 ± 1.24	**0.005**
Pnet (µmol O_2 L^{-1} h^{-1})*	-0.16 ± 0.14	-0.26 ± 0.07	**0.003**
BOD (µmol O_2 L^{-1} h^{-1})	0.25 ± 0.10	0.29 ± 0.08	0.369
Pgross (µmol O_2 L^{-1} h^{-1})	0.09 ± 0.10	0.02 ± 0.09	0.094
N_2 fixation (nmol C_2H_4 L^{-1} h^{-1})*	0.34 ± 0.18	0.50 ± 0.48	1.000

Mean weekly measurements of status parameters were variable over time (Fig. 3.1). Daily PAR increased from January to April (i.e. mixed season), and decreased again from September to November (i.e. stratified season) (Fig. 3.1 a, b). Temperature was stable throughout the mixed season (22.1 – 22.9 °C), but had increased to a maximum of 27.5 °C in early September followed by a decrease to 24.7 °C until the end of November. Inorganic nutrient and Chl a concentrations were all significantly lower during the stratified than during the mixed season (Fig. 3.1 c, d; Table 3.1, $p < 0.003$), while DOC was significantly higher during the stratified season than during the mixed season. POC concentrations showed no significant differences between seasons, while PN was significantly

lower in the stratified season (Fig. 3.1 e, f), causing a significantly higher POC:PN ratio. The DIN:PO_4^{3-} ratio was not significantly different between seasons.

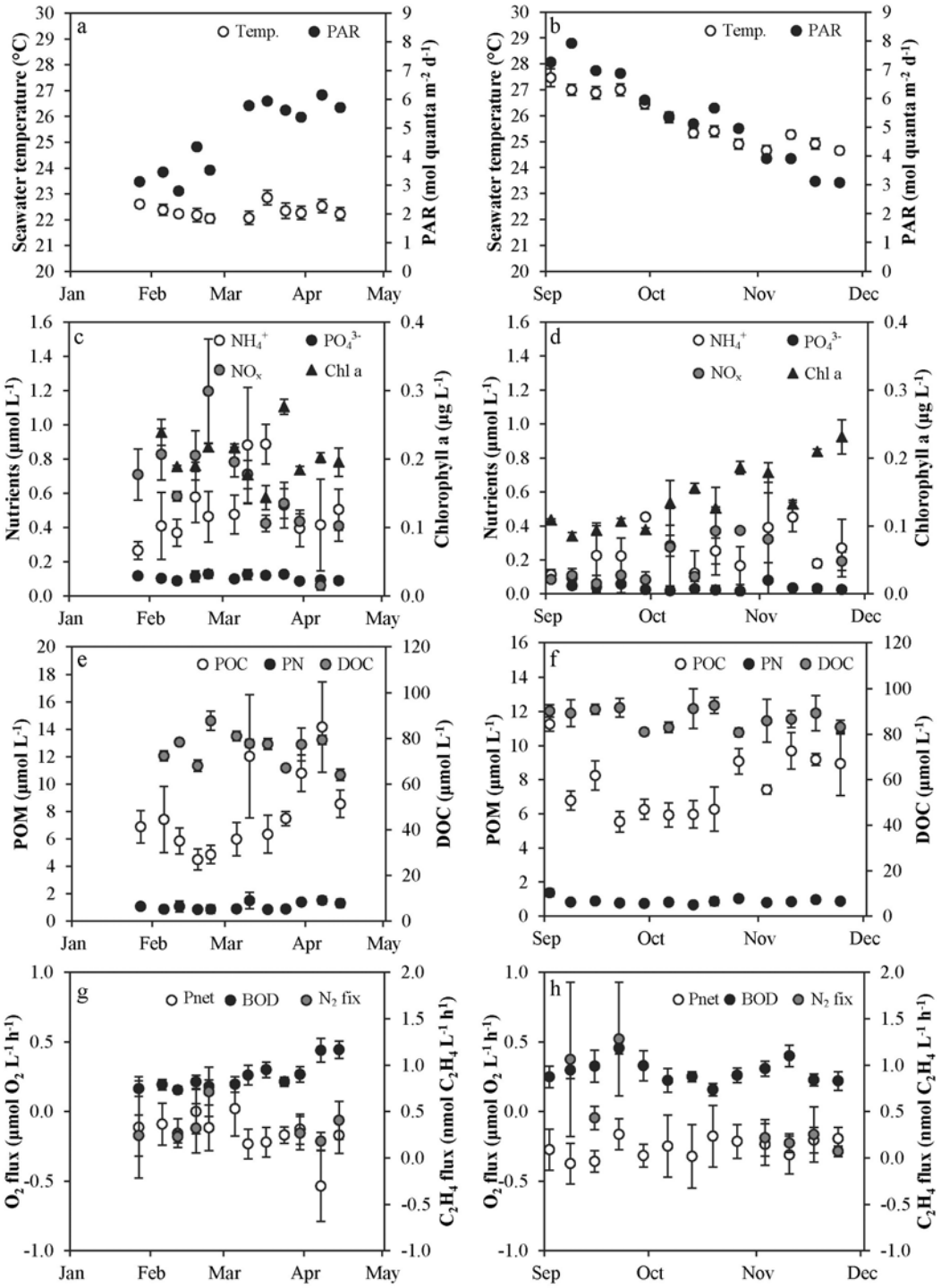

Figure 3.1. Mean weekly status and process parameters during mixed (a, c, e, g) and stratified (b, d, f, h) seasons. Values given as mean ± SD. Temp. = water temperature, PAR = photosynthetically active radiation, NO_x = NO_2^- + NO_3^-, POM = particulate organic matter, POC = particulate organic carbon, PN = particulate nitrogen, DOC = dissolved organic carbon, PP = net primary production, BOD = biological oxygen demand, N_2 fix = N_2 fixation.

Picoplankton community composition

There was a clear seasonal shift in the photosynthetic picoplankton community from the mixed to the stratified season (Fig. 3.2). *Prochlorococcus* mean abundance increased significantly 3-fold, while *Synechococcus* and picoeukaryote mean abundances decreased significantly 2- and 3-fold respectively. Heterotrophic bacteria abundance was very similar between seasons with just a 1.9% increase from the mixed to the stratified season.

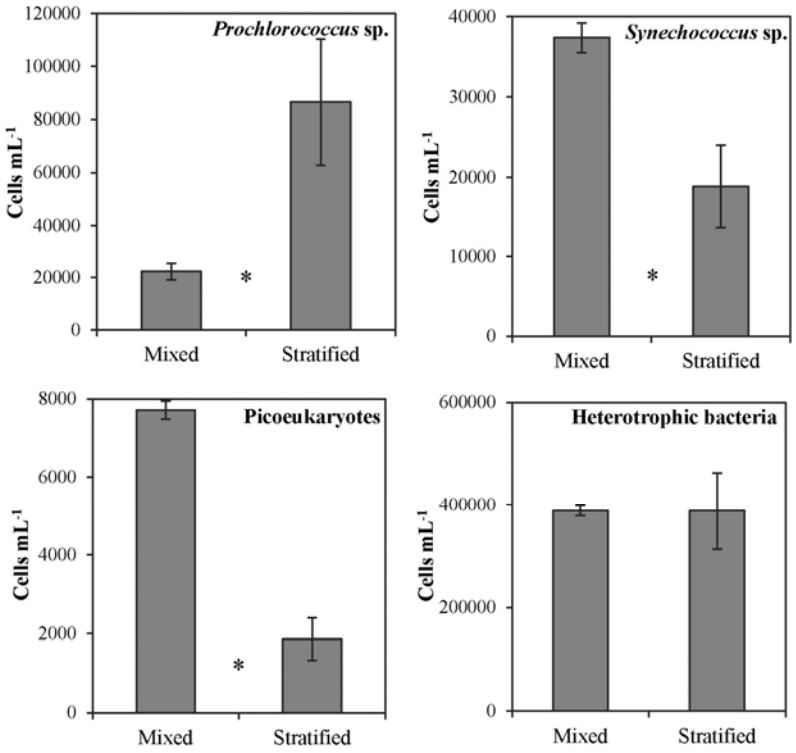

Figure 3.2. Picoplankton group abundances in the mixed and stratified seasons. Values given as mean ± SD. * indicates a significant difference between seasons (Mann-Whitney U tests $p < 0.05$).

Process parameters

Mean water column Pnet was negative in all weeks (-0.54 to -0.0001 µmol O_2 L^{-1} h^{-1}), except on 18 Feb.: 0.02 µmol O_2 L^{-1} h^{-1}, and significantly lower during the stratified season than the mixed season (Fig. 3.1 g, h; Table 3.1). BOD ranged from 0.15 to 0.46 µmol O_2 L^{-1} h^{-1} and was not significantly different between seasons. Estimates of Pgross were similar for the mixed and stratified seasons with averages of 0.09 and 0.02 µmol O_2 L^{-1} h^{-1} respectively, which were not significantly different. Weekly mean N_2 fixation ranged from 0.18 to 0.71 and 0.07 to 1.28 nmol C_2H_4 L^{-1} h^{-1} in the mixed and stratified seasons respectively (Fig. 3.1 g, h). Although mean N_2 fixation on 8 and 22 Sep., during stratification, was substantially higher than during the mixed season, overall there was no significant seasonal difference.

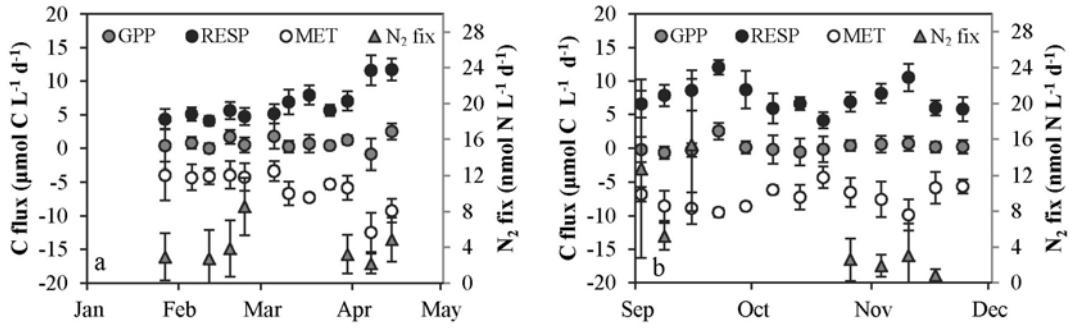

Figure 3.3. Mean daily metabolic carbon fluxes and N_2 fixation during mixed (a) and stratified (b) seasons. Values given as mean ± SD. GPP = gross primary production, RESP = community respiration, MET = metabolic balance over the day = GPP-RESP, N_2 fix = N_2 fixation

Correlation analyses

Analyses for both seasons combined identified strong negative correlations between temperature and each of the inorganic nutrient concentrations (Appendix III - Table 1); showing the expected effect of the mixing-stratification cycle. Chl a, Pnet, and Pgross were all negatively correlated with temperature and positively correlated with two of the inorganic nutrients (Chl a: PO_4^{3-} and NO_x; Pnet: PO_4^{3-} and NO_x; Pgross: NH_4^+ and NO_x). Interestingly, daily PAR was negatively correlated with both Chl a, and Pnet. During the mixed season, correlation analyses revealed that Pnet was positively correlated with NO_x, while lacking correlations with the other nutrients (Appendix III - Table 2). BOD was positively correlated with POC and N_2 fixation was positively correlated with Pgross. In the stratified season, both Chl a and Pnet were positively correlated with NO_x, while the other nutrients were not correlated to either Pnet, or Pgross (Appendix III - Table 3). Finally, N_2 fixation was positively correlated to both temperature and DOC during stratification.

Discussion

Status parameters

PAR, water temperature, and inorganic nutrient concentrations during the mixed and stratified seasons were comparable to previous research at the study site and surrounding waters (Rasheed *et al.* 2002; Naumann *et al.* 2010; Rasheed *et al.* 2012). The mixed season average DIN:PO_4^{3-} ratio was clearly lower than the Redfield ratio for N:P (16:1, Redfield 1958), suggesting N as the limiting nutrient. The average ratio in the stratified season was similar to the Redfield N:P ratio with maximum values between 31 and 33 in 3 nonconsecutive weeks in October and November, suggesting that inorganic N and P availability in that season was on average balanced, but there were times when P could have been limiting. Previous studies have found both N and P as limiting factors for primary production in the northern Red Sea (Stihl *et al.* 2001; Mackey *et al.* 2007; Suggett *et al.* 2009).

Average POC:PN ratios in both seasons were higher than Redfield (106:16), suggesting that the particulate organic matter (POM) in the water column was impoverished in N throughout the year, particularly so during the stratified season when PN concentrations were significantly reduced. The dominant source of POM in coral reef-surrounding waters is often mucus released by hard corals (Johannes 1967; Naumann *et al.* 2012a). Mucus POC or PN release by the dominant scleractinian corals in the studied reef is constant over the year (Naumann *et al.* 2010). However, the average POC:PN ratio of coral mucus (12 ± 1), is far higher than the ratio found for the water column here, indicating that a large fraction of water column PN originated from different sources which may be affected by seasonality. This is confirmed by Hadas *et al.* (2009) who found the majority of water column PN in a Gulf of Aqaba reef to consist of, or be produced by, pelagic prokaryotes. DOC is more abundantly released by benthic coral reef algae than by co-occurring hard corals (Haas *et al.* 2013; Müller *et al.* 2014). DOC release by turf algae and the algal genus *Peyssonnelia* in the studied reef is higher during the stratified than during the mixed season (Haas *et al.* 2010), potentially due to increased temperature. A positive relation between DOC release and temperature is common in marine macrophytes (e.g. Maher and Eyre 2010). Increased benthic release could explain the increased water column DOC concentration found during the stratified season.

Primary production

The Pnet and BOD measurements can be recalculated to metabolic carbon fluxes per day by the following equations, assuming photosynthetic and respiratory quotients of 1.4 and 1.1, respectively (McKinnon *et al.* 2013). Daily gross primary production (GPP) = (Pnet + BOD) x h of daylight; daily respiration (RESP) = BOD x 24 h; daily metabolic balance (MET) = GPP – RESP All carbon fluxes are in µmol C L^{-1} d^{-1}. GPP was relatively stable within each season, but significantly higher in the mixed than in the stratified season, while mean RESP was not significantly different between seasons (Fig. 3.3; Fig. 3.4). MET was always negative and significantly lower in the stratified than the mixed season (Fig. 3.4), indicating that the water column as a whole acted net heterotrophically, particularly so during the stratified season. Coral reef planktonic communities are often described as net heterotroph and are likely fuelled by a steady supply of organic matter released from the reef benthos (Naumann *et al.* 2012b; Haas *et al.* 2013). The planktonic GPP measured in the mixed and stratified seasons falls in the range of other reef-surrounding waters worldwide (Table 3.2). Mean seasonal water column total organic C (TOC = POC + DOC) was 83.5 and 95.1 µmol L^{-1} for the mixed and stratified seasons respectively. Relating seasonal TOC to MET resulted in similar net daily planktonic C turnover of 7.3 ± 3.2, and 7.7 ± 1.8 % d^{-1} during the mixed and stratified seasons respectively (Fig. 3.4).

The picoeukaryote to *Prochlorococcus* sp. change in the phytoplankton community from the mixed to the stratified season was similar to a previous study (Al Najjar *et al.* 2007). This change in phytoplankton composition may explain the negative correlations found between PAR and Chl a, and Pnet. While PAR reached maximum values in the beginning of the stratified season, picoeukaryote

abundance was substantially decreased compared to the mixed season. Since picoeukaryotes contain up to two orders of magnitude more Chl a per cell than *Prochlorococcus* sp. (Davey *et al.* 2008), and are capable of primary production rates up to two orders of magnitude higher than *Prochlorococcus* sp. per cell (Worden *et al.* 2004), both Chl a and Pnet were reduced during highest PAR.

Table 3.2. Comparison of GPP (daily gross primary production) rates from this study and literature. †recalculated from O_2 values using a photosynthetic quotient of 1.4 (McKinnon *et al.* 2013). *extrapolated to per day assuming 12 hours of daylight.

Location	GPP (µmol C L^{-1} d^{-1})	Reference
Gulf of Aqaba	0.00 - 2.54†	This study
Gulf of Aqaba	0.05 - 3.38*	Levanon-Spanier *et al.* 1979
Northern Red Sea	0.02 - 3.00*	Qurban *et al.* 2014
Great Barrier Reef, Australia	0.80 - 3.33	Sorokin 1995
Vietnam	0.15 - 3.00	Tac-An *et al.* 2013
Hawaii	2.01 ± 0.17† (mean ± SE)	Johnson and Wiegner 2014
New Caledonia	0.47 ± 0.05 (mean ± SE)	Toretton *et al.* 2010

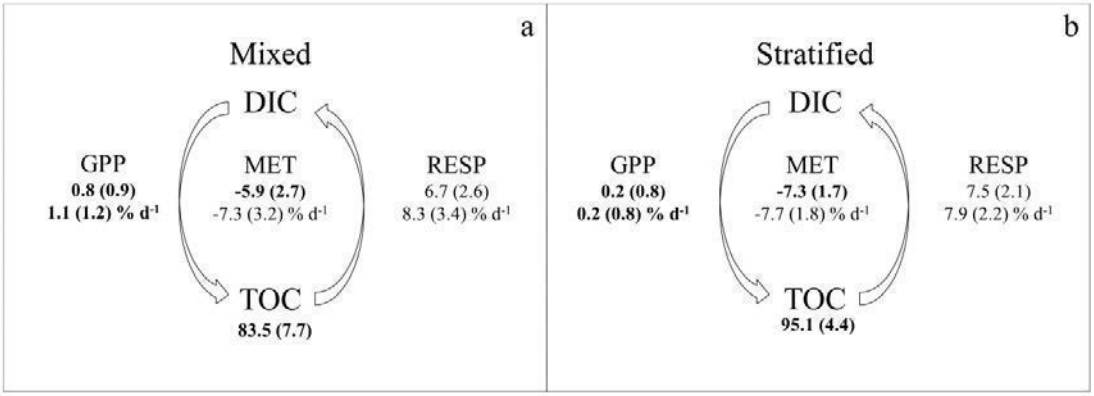

Figure 3.4. Mean seasonal daily planktonic carbon balance in mixed (a) and stratified (b) seasons. GPP = gross primary production, RESP = community respiration, MET = daily metabolic balance = GPP - RESP. Values given as mean (SD). GPP, RESP, MET in µmol C L^{-1} d^{-1}. TOC = total organic carbon in the water column = POC + DOC, TOC in µmol C L^{-1} DIC = dissolved inorganic carbon. Below each C flux is the percentage d^{-1} (mean (SD)) it constitutes of TOC. Bold indicates a significant difference between seasons (Mann-Whitney U tests $p < 0.05$).

Pgross and PAR showed no correlation, while Pgross and NH_4^+, and NO_x were positively correlated, indicating that inorganic N, rather than PAR, was limiting photosynthesis in these well-lit, but oligotrophic, waters. This is supported by similar findings for the northern Red Sea (Qurban *et al.* 2014). Although DIN:PO_4^{3-} ratios in the stratified season often exceeded Redfield with values up to 33, no correlations were found between Pgross and PO_4^{3-}. This may be explained by the size of *Prochlorococcus* which is far smaller than that of picoeukaryotes. Their smaller size with theoretically increased uptake efficiency due to allometrically higher surface area to volume ratio, may have allowed

them to maintain photosynthesis under extremely low phosphorus (P) availability (Finkel *et al.* 2009). However, Marañón *et al.* (2013) found this surface area to volume relation to be lacking over a wide range of phytoplankton sizes (10^{-2} - 10^{7} µm). In addition, primary production by various kinds of phytoplankton may have a wide range of optimal N:P ratios, often strongly exceeding the classic Redfield ratio of 16:1 (Klausmeier *et al.* 2004).

N_2 fixation

The hourly C_2H_4 fluxes can be converted into daily N fluxes by multiplying by 24 h, dividing according to the theoretical conservative C_2H_4:N_2 ratio of 4:1 (Mulholland *et al.* 2004), and multiplying by 2 to convert N_2 to N, resulting in fluxes of nmol N L^{-1} d^{-1}. Daily N_2 fixation rates in both seasons ranged from 0.86 to 15.39 nmol N L^{-1} d^{-1} and average N_2 fixation was similar between seasons: 4.04 ± 2.17 and 5.96 ± 5.74 nmol N L^{-1} d^{-1} (mean ± SD) for mixed and stratified seasons, respectively (Fig. 3.3). Assuming the Redfield C:N ratio, planktonic water column N_2 fixation had, on average, the potential to contribute 3.42 % of N needed for GPP during the mixed season. The average potential contribution during stratification was substantially higher as N_2 fixation produced 20.84 % of the N demand by GPP.

N_2 fixation rates measured by this study were within the range found for planktonic communities from different locations worldwide (Table 3.3). Our maximum rates appear high compared to most literature values, but this is mainly due to two high values measured in September (12.74, 15.38 nmol N L^{-1} d^{-1}). The other weekly mean values in the present study have an average of 3.43 ± 1.88 nmol N L^{-1} d^{-1} which falls within the range of most values from literature (0.01 - 6.24 nmol N L^{-1} d^{-1}). The measurements from the tropical Atlantic Ocean (up to 12.44 nmol N L^{-1} d^{-1}, Großkopf *et al.* 2012) indicate that high rates as found in September are possible in comparable oligotrophic conditions. Moreover, the high September N_2 fixation rates coincided with lowest DIN concentrations of all weeks measured (0.20 - 0.29 µmol L^{-1}). N_2 fixation is energy-costly, and many diazotrophs can increase their N_2 fixation in times of inorganic/organic N scarcity (Mulholland *et al.* 2001).

Table 3.3. Comparison of daily dinitrogen (N_2) fixation rates from this study and literature. †recalculated from C_2H_4 production rates using a C_2H_4:N_2 ratio of 4:1 (Mulholland *et al.* 2004).

Location	N_2 fixation (nmol N L^{-1} d^{-1})	Reference
Gulf of Aqaba	0.86 – 15.39†	This study
Gulf of Aqaba	0.01 - 1.90	Foster *et al.* 2009
Gulf of Aqaba	~ 0.40	Rahav *et al.* 2013
Hawaii	0.70 - 2.21	Dore *et al.* 2002
Hawaii	0.96 - 6.24	Wilson *et al.* 2012
North East Pacific Ocean	0.15 - 0.31	Needoba *et al.* 2007
Tropical Atlantic Ocean	~ 0.96	Falcon *et al.* 2004
Tropical Atlantic Ocean	0.02 - 12.44	Großkopf *et al.* 2012

Rahav *et al.* (2013) found PO_4^{3-} amendments to planktonic N_2 fixation incubations in the Gulf of Aqaba to enhance N_2 fixation. However, Foster *et al.* (2009) did not detect P limitation of N_2 fixation and attributed this to the relatively small size of the N_2 fixing microbes in the Gulf of Aqaba, allowing maintenance of N_2 fixation at very low P availability due to the same allometric relationship as discussed above. We did not find correlations between N_2 fixation and PO_4^{3-} which could indicate such a limitation. However, nutrient limitation of planktonic processes may well be a more complicated subject than indicated by the frequent use of the canonical Redfield ratio (Klausmeier *et al.* 2004).

Rahav *et al.* (2013) also found evidence for a potential transition from autotrophic to heterotrophic diazotrophs between the mixed and stratified seasons. The positive correlations found in the present study between N_2 fixation and Pgross in the mixed season, and N_2 fixation and DOC concentration during stratification lend additional support to such a transition. The potential N contribution by N_2 fixation to primary production was more than six times higher in the stratified season than in the mixed season, indicating N_2 fixation as a possible substantial source of N-nutrients to the autotrophic community as a whole during extremely oligotrophic times in the Gulf of Aqaba. The contribution percentages calculated in the present study are comparable to those under comparable oligotrophic scenarios in other regions (Montoya *et al.* 2004; White *et al.* 2007).

Ecological implications

Seasonal primary production in the water column of the Gulf of Aqaba appears to be primarily regulated by inorganic N availability. As inorganic N concentrations decline due to stratification, GPP decreases substantially resulting in a more heterotrophic daily net C balance coinciding with a shift in the pico-phytoplankton community. While MET was significantly more heterotrophic during stratification, daily net carbon turnover was similar between seasons because of increased DOC concentrations. The maintenance of biological activity in the water column due to increased DOC availability in times of reduced GPP underlines the importance of the microbial loop in planktonic tropho-dynamics in these waters (Azam and Malfatti 2007, Nelson *et al.* 2011).

In addition to this change in the C budget, indications of a potential shift in the N_2 fixation community from its autotrophic to its heterotrophic component, as postulated by Rahav *et al.* (2013), were found. Causes for this shift could be the decline in inorganic nutrients, as well as, again, the increased DOC concentration in the water, giving a competitive advantage to the heterotrophic fraction (Suggett *et al.* 2009). This increase in DOC may be primarily attributable to increased release by benthic turf- and macroalgae (Haas *et al.* 2013; Müller *et al.* 2014). Algal-derived organic matter has also been indicated in promoting a more heterotrophic planktonic community than that derived from scleractinian corals (Haas *et al.* 2011; Nelson *et al.* 2013). During stratification, when GPP is strongly nutrient limited and significantly reduced, N_2 fixation has the potential to contribute a substantial fraction of the N needed to maintain the reduced primary production in the water column.

This study is a first investigation of regulatory effects by key status parameters on both primary production and N_2 fixation as well as phytoplankton community composition in waters surrounding a high latitude coral reef and shows that the seasonality in status parameters coincided with strong changes in the phytoplanktonic community composition over the year with subsequent consequences for planktonic primary production. While indications were found for a similar community change in diazotrophs, N_2 fixation was maintained at comparable levels in both seasons, potentially being an important source of nitrogen to planktonic primary production. DOC appears to play an important role in dynamics of planktonic C and N fluxes. Further investigation into DOC fluxes through coral reefs is warranted to unravel its effect on energy and nutrient cycles in coral reefs and their surrounding waters. The findings presented here on which status parameters play a controlling role in the production of organic C and N-nutrients in reef surrounding waters can be applied to lower latitude coral reefs where the more stable environmental conditions make the disentanglement of driving status parameters more complicated.

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Chapter 4
Scleractinian corals: the ecosystem engineers

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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Main text

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 (Moberg and Folke 1999). 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 (Wild *et al.* 2011). These sessile cnidarians owe their evolutionary success to an endosymbiosis with photosynthetic dinoflagellate microalgae of the genus *Symbiodinium* (commonly referred to as zooxanthellae) (Dubinsky and Jokiel 1994; Stambler 2011) 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 (Wild *et al.* 2004; de Goeij *et al.* 2013).

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) (Grottoli *et al.* 2006). Additionally, corals have evolved strategies to exploit any source of inorganic and organic nitrogen (N) (Cardini *et al.* 2014), enabling them to survive in oligotrophic tropical waters where N is the most limiting nutrient to primary productivity (D'Elia and Wiebe 1990; Eyre *et al.* 2008). However, the abundance of symbiotic zooxanthellae within the coral host is limited by N concentrations (Falkowski *et al.* 1993; Dubinsky and Jokiel 1994). N deficiency and starvation of zooxanthellae cause severe depletion of the main N-containing photopigment (Hoegh-Guldberg and Smith 1989; Jiang *et al.* 2014), 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 (Szmant-Froelich 1983).

Besides endosymbiotic eukaryotic algae, hard corals are also associated with a diverse array of prokaryotes (Rohwer *et al.* 2002). The prokaryote-dinoflagellate-coral assemblage is often referred to as the coral holobiont (Rohwer *et al.* 2002). In the resulting host-microbe ecosystem, the interactions among the partners determine the functioning and ecological success of the whole group of organisms (McFall-Ngai *et al.* 2013). Evidence is accumulating that among these prokaryotic communities dinitrogen (N_2) fixing microorganisms (termed diazotrophs) form highly specific associations with their cnidarian hosts (Shashar *et al.* 1994; Lesser *et al.* 2004; Lesser *et al.* 2007; Olson *et al.* 2009; Lema *et al.* 2012; Lema *et al.* 2014b). However, the ecological significance of N_2 fixation in sustaining the high gross primary productivity of hard corals has not yet been evaluated.

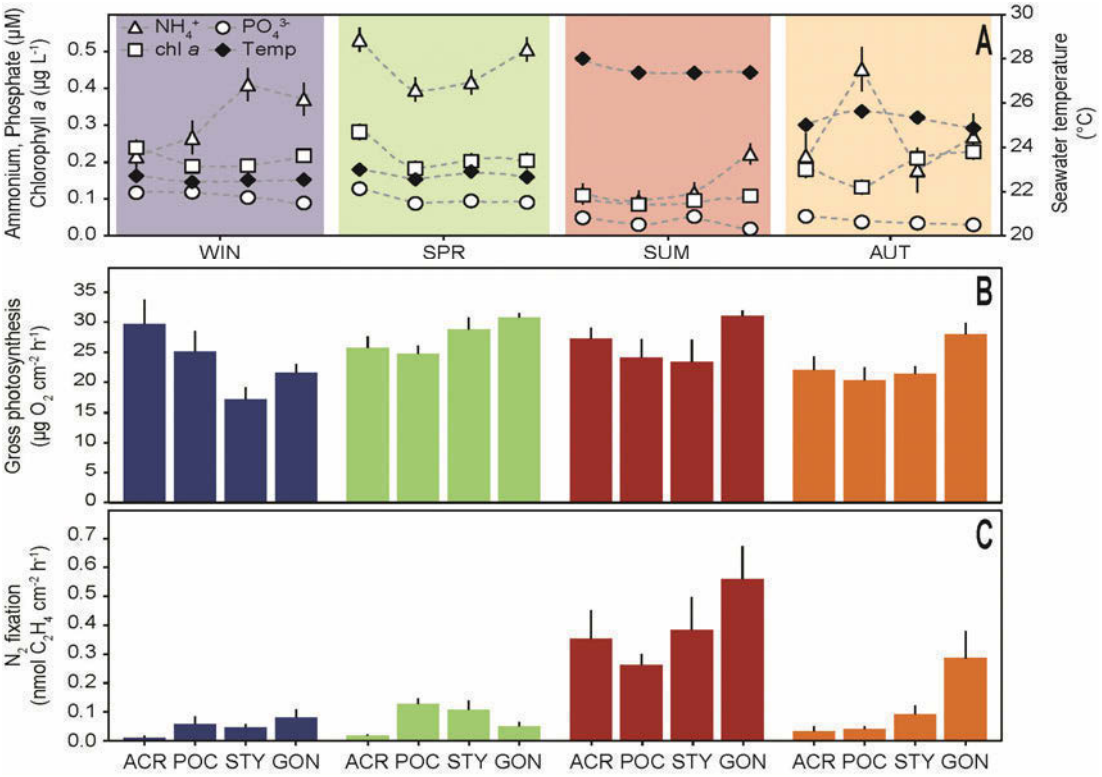

Figure 4.1. Seasonal environmental conditions with corresponding primary productivity and dinitrogen fixation in corals. (A) Seasonal weekly averages of the main environmental variables measured at the sampling location are shown. (B) Gross photosynthesis and (C) N_2 fixation of *Acropora* (ACR), *Pocillopora* (POC), *Stylophora* (STY) and *Goniastrea* (GON) are presented here as means (n=8) ± s.e.m.. See Appendix IV 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).

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, Appendix IV - Fig. 1). This leads to inorganic N concentrations which are one order of magnitude lower in summer compared to spring (Appendix IV - Table 1). 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.

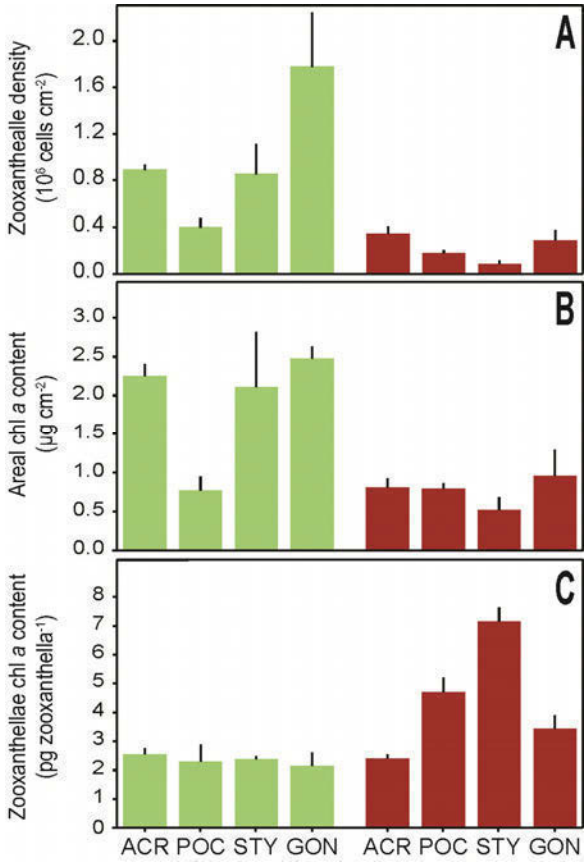

Figure 4.2. Seasonal zooxanthellae and photopigment content in corals. Shown are (A) zooxanthellae density, (B) areal chl a content, and (C) zooxanthellae chl a content. Colours represent spring (green) and summer (red). See Appendix IV for the statistical results. Data are presented as means (n=4) ±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

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 (Shashar *et al.* 1994; Lesser *et al.* 2004; Lesser *et al.* 2007; Davey *et al.* 2008; Olson *et al.* 2009; Lema *et al.* 2012; Lema *et al.* 2014b), and N_2 fixation rates measured here are in the range of those reported for corals in the literature (Cardini *et al.* 2014). This highlights diazotrophs as constant members of hard coral holobionts. N_2 fixation significantly increased in all corals during summer (Fig. 4.1, Appendix IV - Table 3, 4). 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, Appendix IV - Table 3 - 6). 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 (Fitt *et al.* 2000; Fitt *et al.* 2001). Moreover, growth efficiency and mitotic index

of the symbiotic algae are temperature-dependent and decrease if temperature exceeds 25°C (Wooldridge 2013). 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.

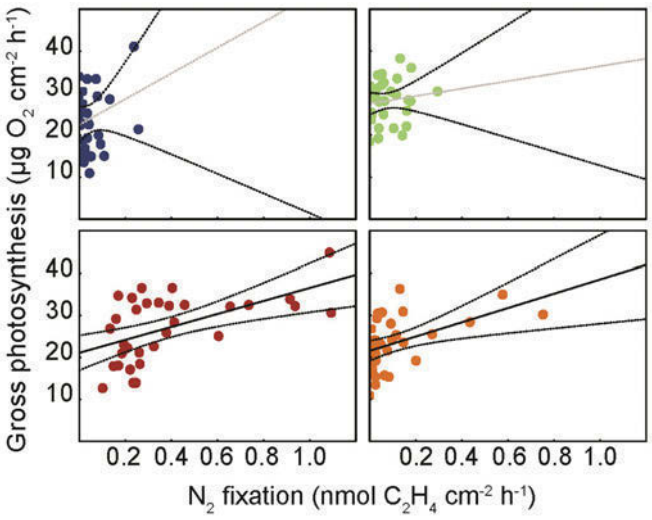

Figure 4.3. Seasonal relationships between N_2 fixation and gross photosynthesis in corals. Data points for each season (n=32) are colour coded in blue (winter), green (spring), red (summer) and orange (autumn). Best-fit linear regression lines (±95% confidence intervals) are solid black if the relationship 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).

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 (Lesser *et al.* 2004). 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 (Lema *et al.* 2012; Lema *et al.* 2014b; Lema *et al.* 2014a), 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.

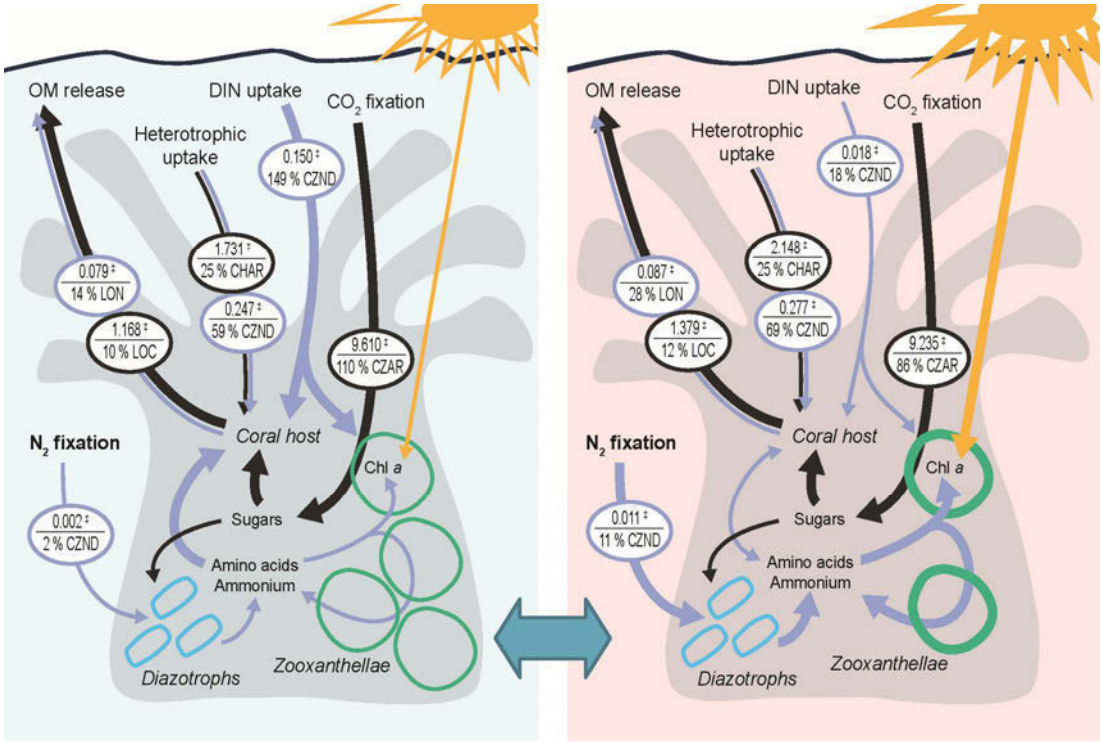

Figure 4.4. C and N flux model of the diazotroph-dinoflagellate-coral symbiosis. Shown are models for spring (left panel) and summer (right panel). Colours represent C (black) and N (blue) fluxes (‡ µmol cm^{-2} d^{-1}), and the width of arrows highlights seasonal differences. Percentages are: contribution 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 in Appendix IV - Table 7.

For tropical coral reefs, it is widely accepted that N is the most limiting nutrient to coral primary productivity (Eyre *et al.* 2008). Phosphorus (P) may also be limiting to coral metabolism (Godinot *et al.* 2011), particularly in the case of an imbalanced nutrient supply with excess inorganic N (e.g., of anthropogenic origin) (Wiedenmann *et al.* 2013). 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 (Appendix IV - Table 1), 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 (Hoegh-Guldberg and Smith 1989), with N deficiency resulting in up to an 86% reduction in zooxanthellae chl *a* content after only one week (Jiang *et al.* 2014). 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.

C and N flux modelling (see Appendix IV - Supplementary methods) 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 (Falkowski *et al.* 1993; Wooldridge 2013). 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 (Fitt *et al.* 2000; Wooldridge 2013). 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, Appendix IV - Table 7).

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, Appendix IV - Fig. 2, Table 7). 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 (Wild *et al.* 2004; de Goeij *et al.* 2013).

Recent research from various reef locations has identified diazotrophic assemblages in different coral species to be spatially and temporally consistent (Lema *et al.* 2012; Lema *et al.* 2014b; Lema *et al.* 2014a). Concurrently, tropical reef systems worldwide typically exhibit extremely low seawater N concentrations, comparable to those measured in summer in the Gulf of Aqaba (Kleypas *et al.* 1999). Further, no lower thresholds for nutrients considered limiting to coral reef growth have been found (Kleypas *et al.* 1999), 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 (Moberg and Folke 1999)) that reef ecosystems provide.

Materials and 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 800 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) (Long *et al.* 2012). 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 (Murphy and Riley 1962; Strickland and 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. 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 (Naumann *et al.* 2009).

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 (Capone 1993; Wilson *et al.* 2012). 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.* (2012). 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 (Shashar *et al.* 1994), 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) (Wilson *et al.* 2012). 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.* (2004). 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 (Herndl and Velimirov 1986; Wild *et al.* 2005; Naumann *et al.* 2010). 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 (Naumann *et al.* 2010) 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 (Pillay *et al.* 2005). 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 (Welschmeyer 1994) 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 (Anderson 2005). 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 (Capone 1993; Montoya *et al.* 1996; Falkowski and Raven 2007), and the proxies chosen are routinely considered accurate surrogates for measurements of the latter rates in hard corals.

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Chapter 5
Soft corals: the competitors for space

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.

Materials and methods

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.

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.

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.

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).

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)

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..

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)

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.

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)

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 5.4. 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	Temperature	Light
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	

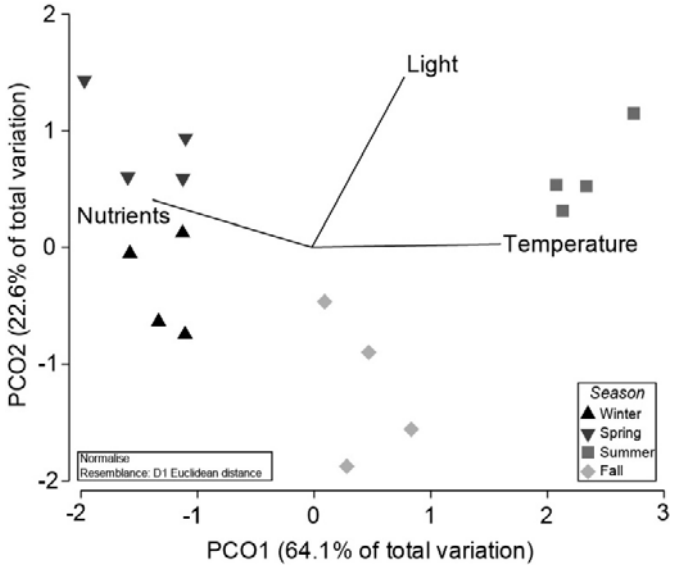

Figure 5.1. Principle coordinates analysis performed on Euclidian distance matrix and normalized data for the different environmental variables (temperature, light, inorganic nutrients) measured in seawater samples from 10 m water depth once a week during four different seasons (winter, spring, summer,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

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.

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
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
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

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.

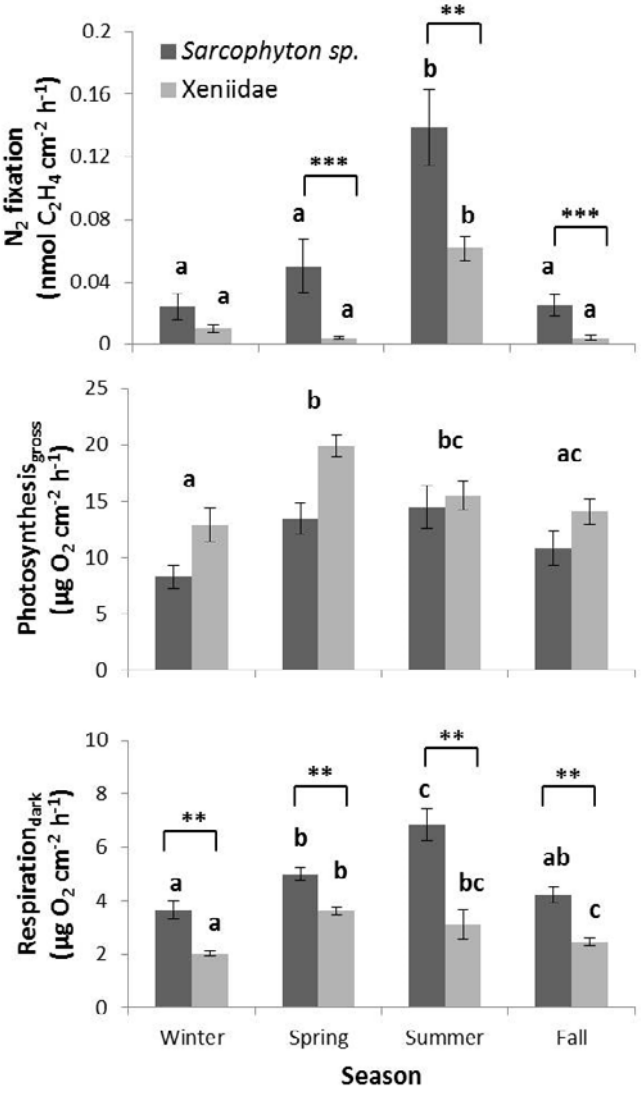

Figure 5.2. Rates of N_2 fixation, P_{gross} and R for *Sarcophyton* sp. and Xeniidae measured during four different seasons (winter, spring, summer, fall) in 2013. Values are given as mean ± SE (n=8). Asteriks indicate significant differences between the two soft corals during each season (* p<0.05,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

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.

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.

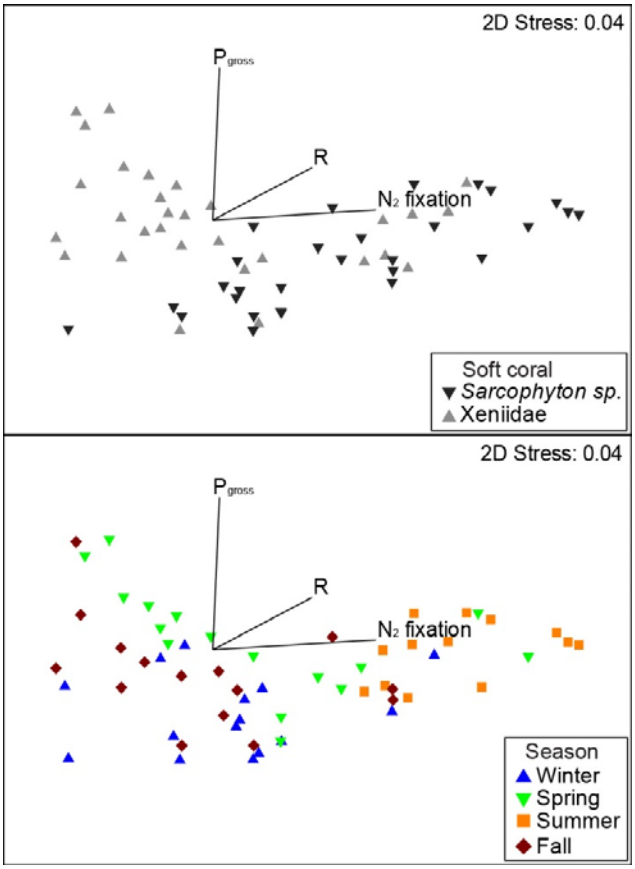

Figure 5.3. Multidimensional scaling plot of N_2 fixation, P_{gross} and R rates for the two soft corals (*Sarcophyton* sp. and Xeniidae) and the four different seasons (winter, spring, summer, fall). Analysis was performed on Bray Curtis similarities of square root transformed data.

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 6
Consolidated substrates: the three-dimensional framework

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.

Materials and 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 6.1). 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 800 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 week 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.

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 6.1). 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 6.1). 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 6.1).

Table 6.1. 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

Dinitrogen fixation

N_2 fixation activity varied significantly by substrate, season, and an interaction between the two factors (Fig. 6.1, Appendix V - Table 1). 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. 6.1). 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. 6.1). 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 6.2). 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 6.2).

Table 6.2. Linear regression analysis of the influence of key environmental parameters on the P_{gross} and N_2 fixation rates of the three substrates. Data are presented as R-squared values at significance levels of <0.05*, <0.01**, <0.001***. Bold values indicate a significant positive linear relationship and italicized 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**

N_2 fixation activity also varied significantly by time of day (Fig. 6.2, Appendix V - Table 2). *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. 6.2). 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. 6.2).

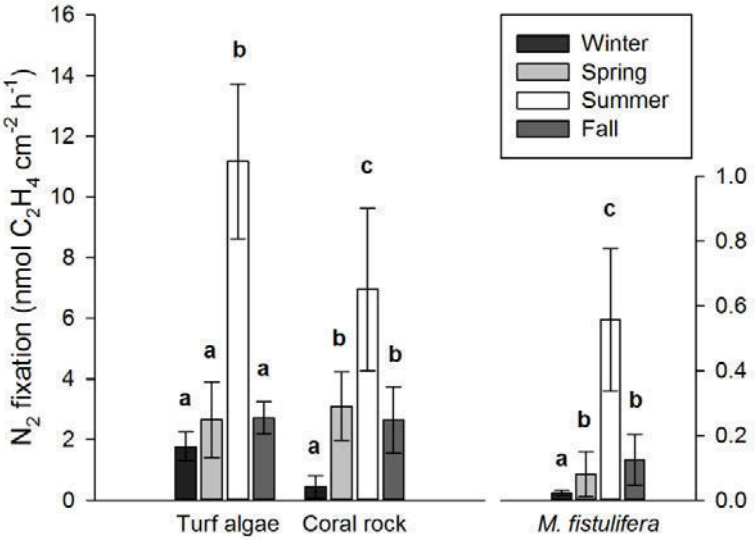

Figure 6.1. Mean N_2 fixation rates of the three investigated benthic substrates over the four seasonal periods in 2013; winter (February), spring (April), summer (September), fall (November). Values (acetylene reduction nmol C_2H_4 cm^{-2} h^{-1}) are presented as mean (n = 8) ± SD. Different letters indicate statistical differences within each substrate. Note the different y-axis scale for *Mycale fistulifera*.

Primary productivity

There were significant effects of substrate and season as well as significant interactions between the two factors for all physiological parameters measured (Appendix V - Table 1). 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 6.3). 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 6.3).

Seasonal variations in P_{gross} were less pronounced than the seasonal differences in N_2 fixation (Table 6.3). 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 6.3). 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 6.2). There was little seasonal variation in P_{gross} of coral rock with no significant effect of any of the monitored environmental parameters (Table 6.2).

Table 6.3. 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

Contribution of dinitrogen fixation to primary productivity

Table 6.4. 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 ± 2.50
	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 ± 3.50
	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**

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. 6.3). 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 6.4). 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 6.4).

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 6.5). 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 6.5), 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 6.5). 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).

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.

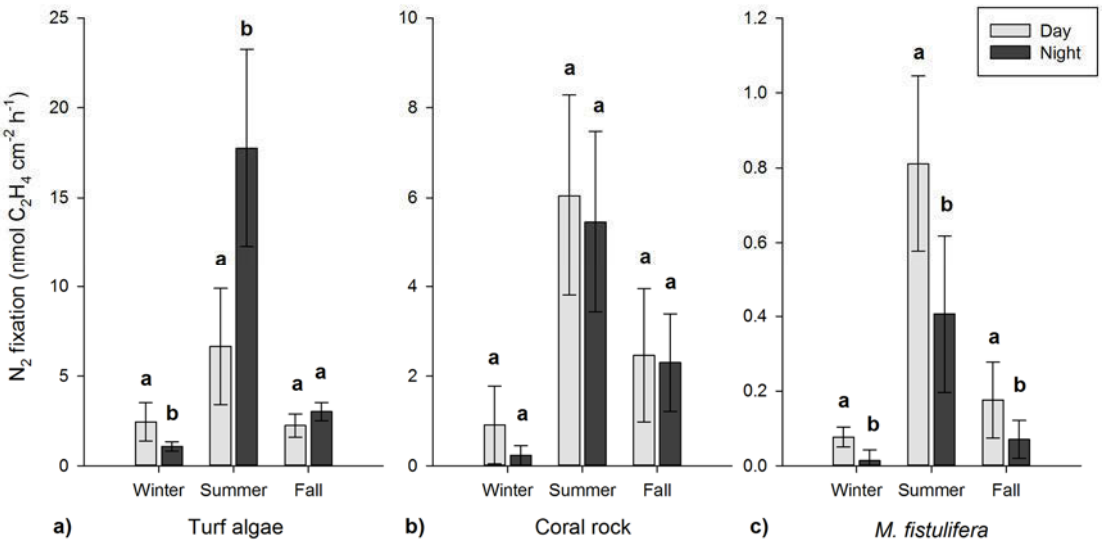

Figure 6.2. Mean day (light bars) and night (dark bars) N_2 fixation rates of a) turf algae, b) coral rock, and c) *Mycale fistulifera* measured over three seasons in 2013 (winter, summer, and fall). Values (acetylene reduction nmol C_2H_4 cm^{-2} h^{-1}) are presented as mean (n = 8) ± SD. Different letters indicate statistical differences within each substrate. Note the changes in scale of the y-axis.

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.

Table 6.5. 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_2 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

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.

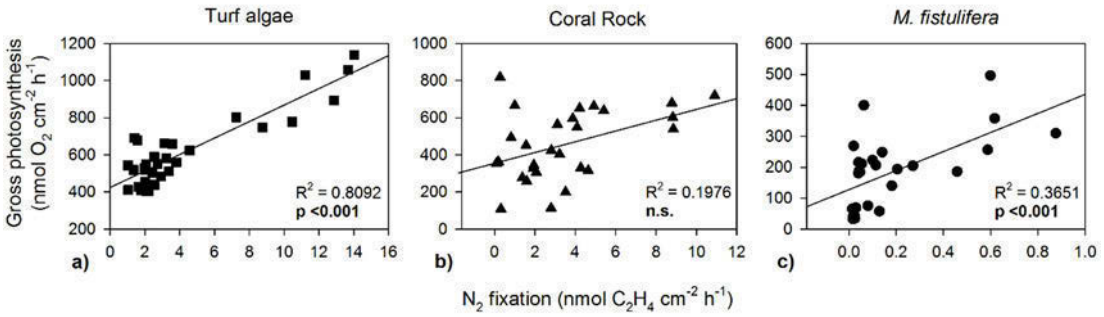

Figure 6.3. Linear regression analysis of N_2 fixation and gross photosynthesis (P_{gross}) rates over all seasons in a) turf algae, b) coral rock, and c) *M. fistulifera*. N_2 fixation is presented as nmol C_2H_4 cm^{-2} h^{-1} and P_{gross} is presented as nmol O_2 cm^{-2} h^{-1}.

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 7
Unconsolidated substrates: the biocatalytic filter system

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 controlling environmental factors for sand-associated N_2 fixation. 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 in *Marine Ecology Progress Series*: Bednarz VN, van Hoytema N, **Cardini U**, Naumann MS, Al-Rshaidat MMD, Wild C. Dinitrogen fixation and primary productivity by carbonate and silicate reef sand communities of the Northern Red Sea.

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).

Table 7.1. 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 ($m^{-2} \times 10^{-12}$)	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})	$3.1 \pm 0.9 \times 10^9$	$1.5 \pm 0.5 \times 10^9$	Schöttner et a. 2011

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 7.1). 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.

Materials and 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 7.2). 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).

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 7.1. 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

Table 7.2. 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)

All monitored environmental key parameters exhibited a strong seasonal patterns (Table 7.2) 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 7.2). 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

Table 7.3. 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			

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. 7.1, Table 7.3). 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.

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. 7.2, Table 7.3). 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 7.4). 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 7.4). 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 7.4). 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 7.3). However, no significant relationship was found between N_2 fixation activity and the key environmental water parameters (Table 7.4). Correlation analysis between N_2 fixation and O_2 fluxes in microbial mats revealed significant positive relationships to both P_{gross} and R (Table 7.4).

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. 7.2). 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 7.4. 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*****

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 7.4). 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 7.1).

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 7.1, 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 7.1). 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.

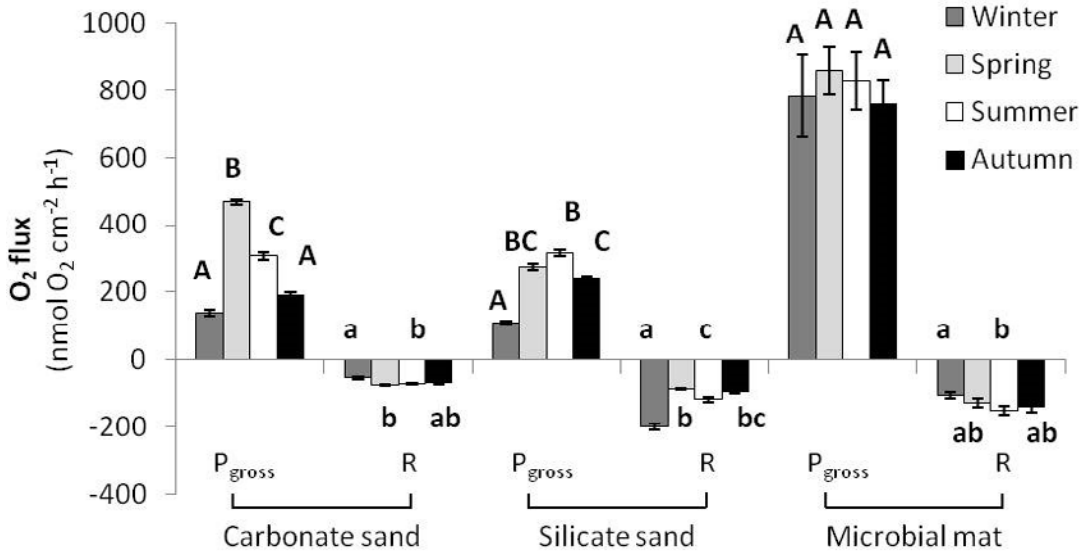

Figure 7.1 P_{gross} and R rates (O_2 fluxes) in carbonate sand, silicate sand and microbial mats during the four seasons (winter, spring, summer, autumn). Values are given as mean (n = 8) ± SE. Different letters indicate significant differences for P_{gross} (A-C) and R (a-c) rates between the four seasons for each substrate type, respectively, based on pair-wise PERMANOVA analysis.

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 7.4). 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.

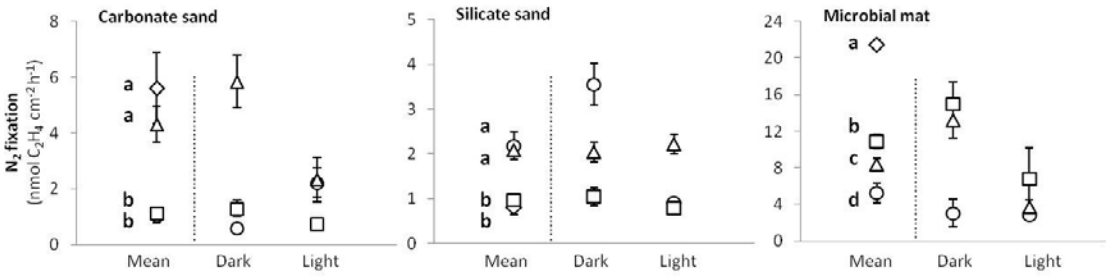

Figure 7.2 Mean N_2 fixation (C_2H_4 production) rates of the different substrates (carbonate sand, silicate sand, microbial mat) measured during winter (circles), spring (diamonds), summer (triangles) and autumn (quadrates) over a 24 h incubation period. For the winter, summer and autumn incubations, 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.

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.

Table 7.5. 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	Reference
Carbonate sands	2.88 ± 0.41	3	0.46 ± 0.07	Red Sea	Present study
	0.04-2.32	4	0.01-0.28	Caribbean	O'Neil & Capone 1989
	0.75-1.95	3	0.12-0.31	GBR, Australia	Capone *et al.* 1992
	19.52 ± 17.50	4	2.34 ± 2.10	Red Sea	Shashar *et al.* 1994
	0.18-1.02	1.8-4.8*	0.03-0.28	French Polynesia	Charpy-Roubaud *et al.* 2001
	-	-	0.10-0.16	Ishigaki Island	Miyajima *et al.* 2001
	0.32	1.6*	0.34	French Polynesia	Charpy-Roubaud & Larkum 2005
	9.76 ± 3.21	4	1.17 ± 0.39	New Caledonia	Charpy *et al.* 2007
	0.03-0.12	3	0.004-0.019	GBR, Australia	Werner *et al.* 2008
Silicate sands	1.52 ± 0.15	3	0.24 ± 0.02	Red Sea	Present study
Microbial mats	11.95 ± 1.16	3	1.91 ± 0.19	Red Sea	Present study
	2.7-47.8	4	0.3-5.7	California	Paerl *et al.* 1993
	0.96	1.6*	0.57	French Polynesia	Charpy-Roubaud & Larkum 2005
	0.59-2.97	4	0.07-0.36	Indian Ocean	Charpy *et al.* 2012

* Conversion factor was empirically determined

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 correlates 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 7.6). 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 7.1, 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.

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Part III

Dinitrogen fixation in hard corals facing climate change

Chapter 8
The effect of ocean warming

Abstract

Different coral holobionts (i.e., the coral-algal-prokaryote symbiosis) exhibit varying thermal sensitivities which may determine if they will adapt to global warming. While it is increasingly recognized that healthy corals are multi-partite symbiotic organisms with prokaryotes potentially providing the plasticity needed to adapt to a changing environment, studies simultaneously investigating the effects of warming on all holobiont members are lacking. Thus, we conducted a thermal stress experiment to understand the role each member plays in influencing the response of the holobiont to thermal stress. We show that exposure to higher temperature affects the main physiological traits of all members (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 temperature (+6 ˚C) resulted in decreased photosynthesis coupled with a drastic increase in light N_2 fixation, particularly in *A. hemprichii* (300 % 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 (200 % compared to controls). Concurrently, both animal hosts displayed impaired organic matter release and picoplankton feeding. These findings suggest that the resilience of the holobiont to warming depends on the cumulative response of its members. In particular, coral-associated diazotrophs show physiological plasticity under high temperature, fixing additional nitrogen that may play a beneficial role during thermal stress events. This functional association can therefore play a fundamental role in the selection of coral species 'winners' and 'losers' when confronted with climate change.

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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 and 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 play a key role in determining coral resistance and resilience to thermal stress. *Symbiodinium* densities generally increase under nutrient replete conditions (Stambler *et al.* 1991; Fabricius 2005), and the addition of nutrients can reduce 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 one of the essential nutrients (mainly nitrogen or phosphorous) is not available to *Symbiodinium* at sufficient concentrations to ensure 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 energy 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 differing capabilities for heterotrophic plasticity (Grottoli *et al.* 2006), and other pathways of nutrient acquisition may be very important for determining coral sensitivity to thermal stress.

Besides their symbiotic algae, corals are associated with a vast diversity of microorganisms. It is increasingly recognized that the functioning of the association among all partners (i.e., coral holobiont) determines the success of hard corals as the primary ecosystem engineers of tropical coral reefs (Barott and Rohwer 2012). Over the last decade, a vast number of studies have characterized the diversity of microbial communities in corals using molecular tools. Several of these studies have 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 healthy corals actually promote their health (Rosenberg *et al.* 2007; Krediet *et al.* 2013). 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; Lesser *et al.* 2007; Olson *et al.* 2009; Lema *et al.* 2012; Lema *et al.* 2014a; Lema *et al.* 2014b). Diazotrophic assemblages in corals are similar among different reef locations, are species-specific, and are established in the early life history stages of their coral host (Lema *et al.* 2012; Lema *et al.* 2014a; Lema *et al.* 2014b). These bacteria may be 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 essential to the functioning of the holobiont under stressful conditions, such as during periods of increased temperatures (Cardini *et al.* 2014).

Here, we investigated the response of two common hard coral holobionts (herein: animal host, *Symbiodinium* and diazotrophs) from the northern Red Sea, *Acropora hemprichii* and *Stylophora pistillata*, to a thermal stress experiment lasting three weeks. The goals of this study were to characterize, quantify and compare in the two holobionts the main ecological traits of the coral host (feeding, calcification, organic matter release), the associated *Symbiodinium* (primary productivity) and the associated diazotrophs (N_2 fixation) in response to thermal stress. Importantly, our study site is characterized by corals with exceptionally high bleaching thresholds, and has thus been suggested to serve as reef refuge 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.

Materials and methods

Sample collection and maintenance

Acropora hemprichii and *Stylophora pistillata* nubbins 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 (at least 5 m apart) using bone cutters, placed in plastic bags and transported back to the laboratory taking care not to cause any tissue abrasion. 16 fragments per species (32 in total) were subsequently glued to ceramic tiles and allowed to recover for two weeks before starting the manipulation experiment. Coral nubbins were randomly assigned and maintained in two aquaria (8 nubbins per species) 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 were analysed 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 and Riley 1962; Strickland and 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. For environmental variables measured during the experiment see details in Appendix VI - Table 1.

Thermal stress experiment

We tested the response of *Stylophora pistillata* and *Acropora hemprichii* to thermal stress by exposing coral nubbins in one of the two aquaria to increasing water temperatures at a rate of 1 °C every two days (Appendix VI - Fig. 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 this 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. Control nubbins received seawater with no temperature manipulation (temperature throughout the experiment = 24.9 ± 0.4 °C).

The absence of treatment replications can result in unexplainable 'aquarium' effect, and variability between replicates has already been reported in coral mesocosm studies (Jokiel *et al.* 2008). This variability is due to differences in the chemical-physical environment in each aquarium caused by the development of algal biomasses and accumulation of detritus (Martin and Gattuso 2009). However, our experiment was a fully cross factorial design in which signs of bleaching occurred exclusively in the thermal treatment aquarium. Aquaria were cleaned twice a week to prevent any development of epiphytes and accumulation of detritus, and we did not find any change in inorganic nutrient concentrations between treatments, with conditions not different from *in situ* (Appendix VI - Table 1). This demonstrates that high seawater renewal rate and aquarium cleaning successfully prevented any 'aquarium' effect.

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 aquarium to ensure maintenance of stable temperature in the individual chambers. Immediately prior to the start of the incubations, ceramic tiles were cleaned of 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 aquarium, 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).

***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 9 mL aliquot was subsequently subsampled, fixed with formaldehyde (final concentration 4 %) and preserved at 4 °C until *Symbiodinium* density analysis. The subsample 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.* 2005). A second 5 mL subsample 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 colour 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 measured by 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 (21:00 - 23:00). DO fluxes were assessed as differences between salinity corrected DO concentrations (incubation time ≈ 90 min) measured with a DO optode sensor and salinity probe (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany). Gross photosynthesis (P_G) was calculated ($P_G = P_N + R_D$, with R_D as positive values) for each specimen, and should be considered a conservative estimate (Al-Horani *et al.* 2003). 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 nubbin surface area.

Coral calcification

Coral light calcification was determined during the incubation for P_N using the alkalinity anomaly technique (Chisholm and 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 0.02 % mercury chloride 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 nubbin surface area.

Coral mucus release

Coral mucus release was quantified 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 sections). The volume of the incubation water was measured and the water was 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 % of the standard value). At the beginning and end of the incubation, samples for DOC (30 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. 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 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. 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 to the incubation volume, incubation period and coral surface area. For the calculation of mucus release rates, 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 analysed 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 (Robinson *et al.* 1997). 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 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 input 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 and in seawater controls, 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 subsamples were transferred to ground-glass stoppered bottles (Wheaton BOD), and initial oxygen concentration in each bottle was determined with an 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 nubbin surface area.

Statistical analysis

Differences in each parameter were assessed using univariate distance-based permutational nonparametric analyses of variance (PERMANOVA) (Anderson 2001). A fully crossed 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 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). 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 with *Symbiodinium* density and areal chlorophyll *a* content decreasing by more than 90 % in the treatment compared to the controls (p < 0.05 and p < 0.001, respectively, PERMANOVA with pair-wise comparisons; Fig. 8.1a,b). *Symbiodinium* cell chlorophyll *a* content also significantly decreased (p < 0.05, PERMANOVA with pair-wise comparisons; Fig. 8.1c).

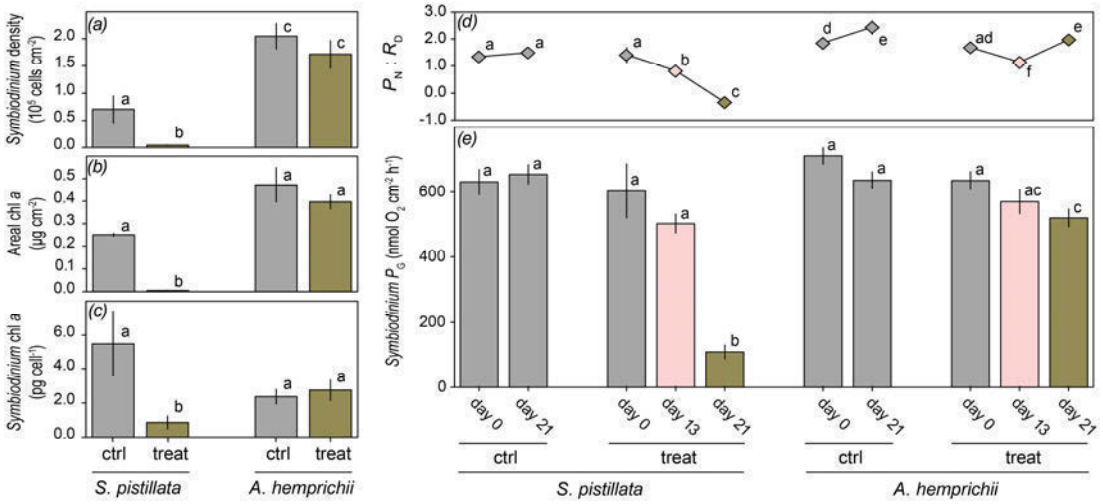

Figure 8.1. *Symbiodinium*-mediated processes. (a) *Symbiodinium* density (cells cm^{-2}), (b) areal chlorophyll *a* (µg cm^{-2}) and (c) *Symbiodinium* chlorophyll *a* (pg cell^{-1}) in the coral nubbins at the end of the experiment. (d) Net photosynthesis versus dark respiration (P_N : R_D) and (e) 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 ± SEM 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; dark green bars, ambient temperature after exposure to 34 °C. Results of the PERMANOVAs are reported in Appendix VI - Table 2, 3.

Conversely, *Acropora hemprichii* did not show any significant loss of *Symbiodinium*, nor of chlorophyll *a* (p > 0.05, PERMANOVA with pair-wise comparisons; Fig. 8.1a,b,c). 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; Fig. 8.1e). 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; Fig. 8.1d). 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; Fig. 8.1d). 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; Fig. 8.1e).

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 not statistically significant (p > 0.05, PERMANOVA with pair-wise comparisons; Fig. 8.2a).

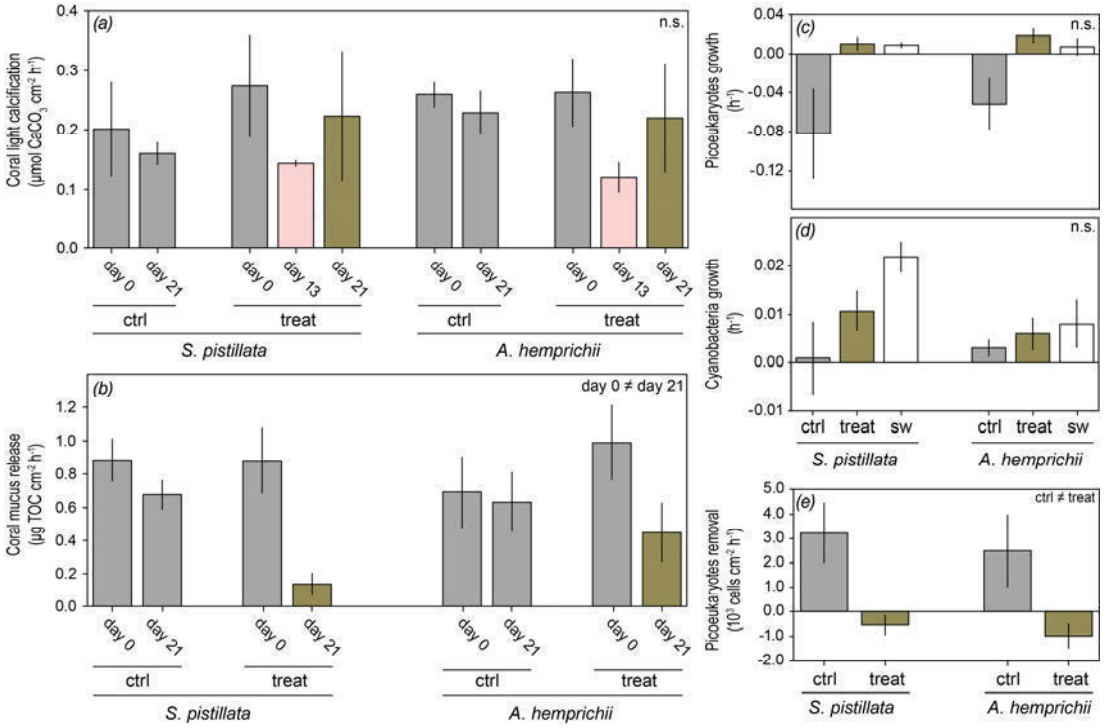

Figure 8.2. Coral-mediated processes. (a) Coral light calcification (µmol $CaCO_3$ cm^{-2} h^{-1}) and (b) mucus release (µg TOC cm^{-2} h^{-1}) at different times during the experiment. (c) Picoeukaryotes growth (h^{-1}) and (d) cyanobacteria growth (h^{-1}) in coral-surrounding water versus seawater controls and (e) picoeukaryotes removal by the corals (cells cm^{-2} h^{-1}) at the end of the experiment. Values are means ± SEM 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; dark green bars, ambient temperature after exposure to 34 °C; white bars, ambient temperature without coral nubbins. Results of the PERMANOVAs are reported in Appendix VI - Table 2, 3.

At the end of the experiment, mucus release rates (as TOC 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; Fig. 8.2b), whereas the interaction term between treatment and time was not significant. This was mainly due to the high variability in DOC release (Appendix VI - Fig. 2). However, a significant decrease in POC release was measured in *S. pistillata* (p < 0.001, PERMANOVA with pair-wise comparisons; Appendix VI - Table 2) but not in *A. hemprichii*.

Picoeukaryotes and cyanobacteria populations had higher average growth rates in incubation chambers with treated corals and in seawater controls than in chambers in which the control nubbins were incubated, potentially indicating decreased feeding by treated corals (Fig. 8.2c,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; Fig. 8.2e), 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; Fig. 8.3a).

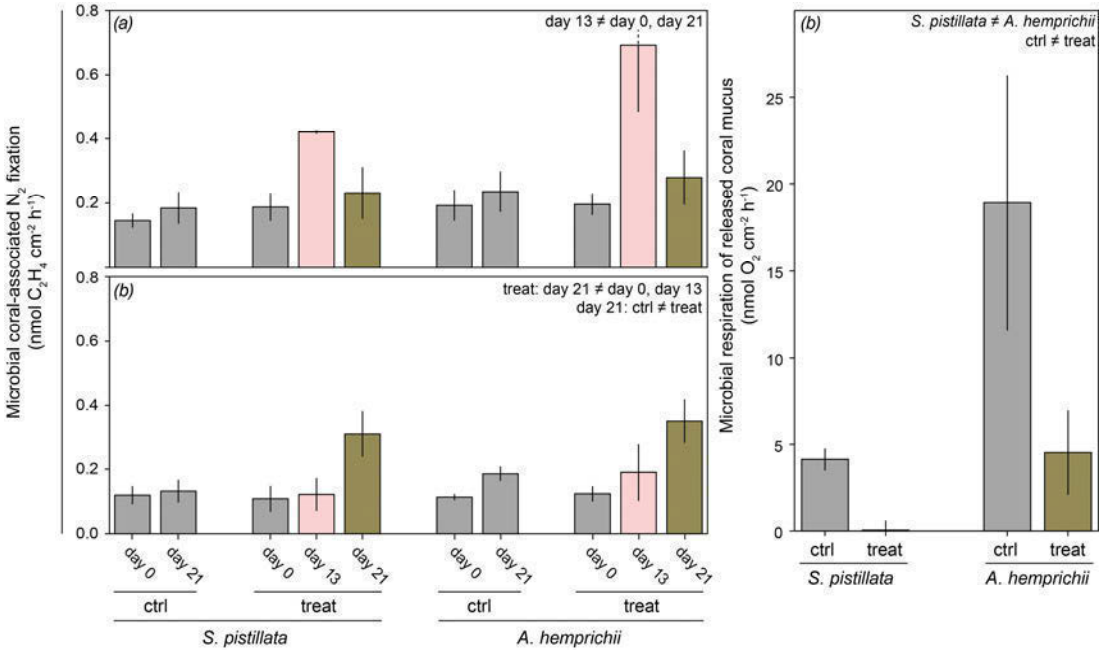

Figure 8.3. Microbe-mediated processes. (a) Light and (b) dark microbial coral-associated N_2 fixation (nmol C_2H_4 cm^{-2} h^{-1}) at different times during the experiment. (c) Microbial respiration of released coral mucus (nmol O_2 cm^{-2} h^{-1}) in coral-surrounding water at the end of the experiment. Values are means ± SEM 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; dark green bars, ambient temperature after exposure to 34 °C. Results of the PERMANOVAs are reported in Appendix VI - Table 2, 3.

This increase was particularly relevant in *A. hempricii*, with light N_2 fixation rates which were 300% compared to the controls. Conversely, light N_2 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; Fig. 8.3b), particularly in *S. pistillata* (200% compared to controls). Concurrently, in the water surrounding treated corals, microbial respiration of released coral mucus decreased significantly ($p < 0.001$, PERMANOVA with pair-wise comparisons; Fig. 8.3c) in response to the decreased organic matter release (Fig. 8.2b).

Discussion

This is the first study simultaneously exploring the effects of warming on all members of the coral holobiont. Our results demonstrate that for each member, 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 affected by thermal stress.

Several earlier studies have demonstrated the susceptibility of the coral-algal symbiosis to high temperatures (Jokiel and Coles 1990; Fitt *et al.* 2001). Consequently, coral reef ecosystems would be 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 and Baird 2000; Manzello *et al.* 2007). Our study supports previous results which have shown that corals from the northern Red Sea are highly resistant to thermal stress 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 of 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 stress. 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 (Appendix VI - Fig. 3) suggesting potential for recovery.

Despite the high resistance of the investigated corals to thermal stress, 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 and Grottoli 2006), while others do so 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 of whether the coral lost its photosymbionts or not, and treated corals removed significantly less picoeukaryotes from the seawater. Heterotrophic plasticity in response to bleaching appears to be highly species-specific, as some species 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 findings of decreased picoeukaryotes removal by treated corals stand in contrast to a previous study that found bleached *S. pistillata* to have increased pico- and nanoplankton grazing rates (Tremblay *et al.* 2012). However, in this study the prey density was concentrated, and corals were incubated in the dark, making it difficult to compare those results with our findings. Overall, our study reveals that the daylight 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 the understanding of the consequences of global warming on coral resilience.

Thermally-stressed corals in this study decreased their release of mucus to the surrounding seawater, particularly in the case of bleached *S. pistillata*. By contrast, other authors have found increased organic matter release by scleractinian corals in response to thermal bleaching (Niggl *et al.* 2009; Tremblay *et al.* 2012). In these studies, the increased production 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 (Wild *et al.* 2011). These speculations are confirmed by our results of microbial respiration of released coral mucus, which decreased 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 for N_2 fixation by coral-associated diazotrophs. While the importance of coral-associated bacteria at 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. Diazotrophic bacteria may play an important role for the coral by providing bioavailable nitrogen in otherwise nutrient-depleted tropical waters (Lesser *et al.* 2007; Lema *et al.* 2012). However, climate change may impair their functioning. In fact, recent studies found coral-associated diazotrophs abundance and diversity to increase at increased temperature (Santos *et al.* 2014), while their activity decrease under high pCO_2 conditions (Rädecker *et al.* 2014). Our results demonstrate for the first time 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 minor increase was also measured in *S. pistillata*. In *A. hemprichii*, this resulted in a nitrogen input from N_2 fixation of 0.16 µg N cm^{-2} d^{-1}, while in control corals the average input was 0.05 µg N cm^{-2} d^{-1}. While these inputs are low compared to what the coral potentially obtains from heterotrophic feeding (Houlbrèque and Ferrier-Pagès 2009), during a bleaching event they may be relevant, particularly if heterotrophy is impaired. Indeed, it is increasingly recognized that the nutrient status of the holobiont impacts 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, thereby having a detrimental effect on the coral resistance to thermal stress (Wiedenmann *et al.* 2013). Conversely, moderate nitrogen enrichment benefits 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. Overall, these results indicate that microbial N_2 fixation can be important to determine the resilience of some coral species to thermal stress. The functional association with diazotrophs can therefore play a fundamental role in the selection of coral species 'winners' and 'losers' when confronted with climate change.

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-sensitive enzyme responsible for N_2 fixation (Berman-Frank *et al.* 2003)) was protected from oxygen inhibition. Conversely, when the coral-algal symbiosis was impaired after the thermal stress (particularly in *S. pistillata*), we measured higher N_2 fixation in the dark, concomitant with higher 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 within 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 9
The effect of ocean acidification

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).

Materials and 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 (2000 L) 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, dark N_2 fixation rates 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.

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 9.1a,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. 9.1c,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.

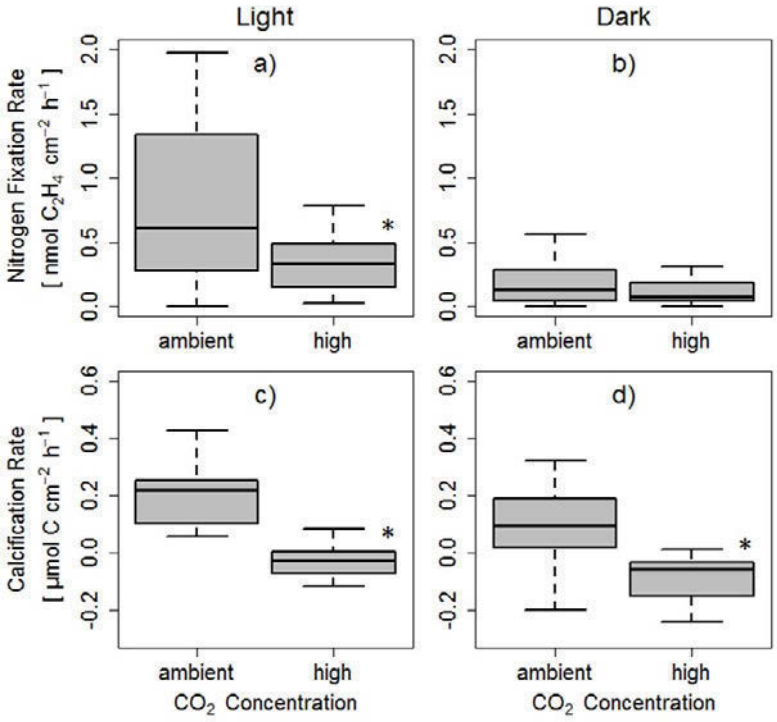

Figure 9.1. Boxplots for N_2 fixation rates (a,b) and calcification rates (c,d) of *S. hystrix* depending on the CO_2 treatment (n = 15 for N_2 fixation rates and n = 13 for calcification rates) for the light (a,c) period and dark (b,d) period. All N_2 fixation rates are expressed as ethylene (C_2H_4) 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.

Since both calcification and N_2 fixation decreased under the ocean acidification scenario, the relationship between these two processes was investigated (Fig. 9.2). 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.

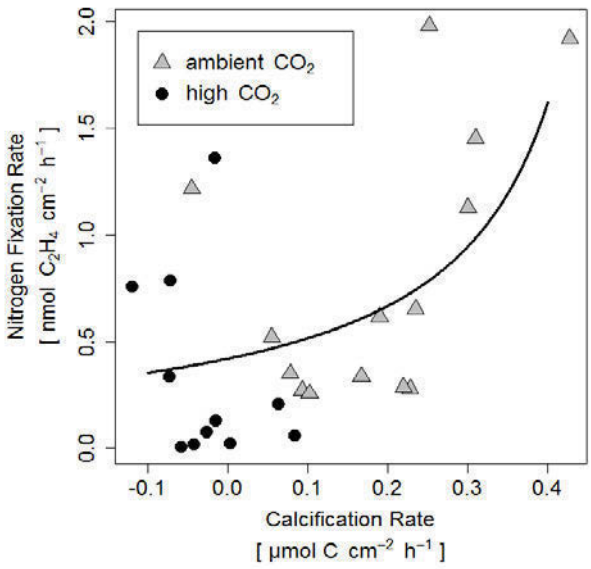

Figure 9.2. Relationship of N_2 fixation rates and calcification rates of *S. hystrix* incubated in the light under high CO_2 (black points) and control conditions (grey triangles). All rates were corrected for seawater controls and normalized to incubation time and coral surface area. Black curve indicates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

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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Part IV

Estimating the contribution of coral reefs to the global nitrogen cycle

Chapter 10
Dinitrogen fixation at the reef ecosystem scale

Abstract

Biological dinitrogen (N_2) fixation (diazotrophy) relieves marine primary producers of nitrogen (N) limitation in the vast oligotrophic zone of the world oceans. N concentrations are particularly low in tropical waters where coral reefs flourish. Here, N represents a key limiting nutrient to these highly productive ecosystems. However, the role and importance of diazotrophy for primary productivity of coral reef ecosystems is still not resolved. In this study, we present areal N_2 and carbon (C) fixation rates in a high spatiotemporal resolution for a fringing reef in the northern Red Sea, based on a combination of physiological laboratory measurements and *in-situ* surveys. We found that diazotrophy was omnipresent in space and time. Contribution of fixed N by planktonic diazotrophs was 20-fold lower compared to the contribution by the reef benthos. Benthic N_2 fixation rates were 0.16 to 0.92 mmol N m^{-2} d^{-1}. These results imply that approximately 10% of the overall net reef primary production was supported by autochthonous input of N derived from N_2 fixation. If our findings can be extrapolated to global reef areas, this suggests reef-wide N_2 fixation rates of 1.60 to 2.58 Tg N yr^{-1}, placing coral reef ecosystems among the benthic communities contributing most to marine fixed N inputs.

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Introduction

Nitrogen (N) is a fundamental component of all living organisms. In particular, N is needed in primary producers in capturing energy through photosynthesis and in building biomass, leading to a tight coupling of the N and the carbon (C) cycle (Gruber and Galloway 2008) and dictating constrains to the flexibility of the ecosystem C:N stoichiometry (Geider and La Roche 2002). However, in unperturbed marine ecosystems bioavailable forms of N are scarce due to low atmospheric inputs and N losses, and primary productivity is often N limited (Vitousek and Howarth 1991). This limitation is of particular significance in coral reef ecosystems, as these are among the most productive ecosystems on Earth, yet exhibiting very low ambient concentrations of dissolved nutrients. Here, biological dinitrogen (N_2) fixation (diazotrophy) is thought to play an important role in replenishing the N reservoir, maintaining the ecosystem productivity and its biological storage of carbon (D'Elia and Wiebe 1990; Capone 1996; O'Neil and Capone 2008). Since the early work by Wiebe *et al.* (1975) many studies found high rates of N_2 fixation associated with several benthic substrates, ranging from sediments and cyanobacterial mats to macroalgae and scleractinian corals (see reviews by O'Neil and Capone (2008) and Cardini *et al.* (2014)). Moreover, after first evidence of diazotroph-coral interactions by Shashar *et al.* (1994a) and the subsequent discovery of diazotrophic cyanobacteria in the tissue of scleractinian corals of the genus Montastraea (Lesser *et al.* 2004), there has been emergent recognition of the potential contribution of N_2-fixing symbioses in corals. While reef sediments and cyanobacterial mats show high rates of N_2 fixation activity (O'Neil and Capone 1989; Capone *et al.* 1992; Shashar *et al.* 1994b; Charpy-Roubaud *et al.* 2001), N_2-fixing coral symbioses may also be responsible of significant inputs of N on an ecosystem level, particularly in high coral coverage areas of the reef. However, rates of N_2 fixation in coral reef habitats are highly variable (O'Neil and Capone 2008) and are potentially affected by global and local anthropogenic disturbances (Cardini *et al.* 2014). Therefore, it is increasingly important to quantify N_2 fixation at the reef scale, and under changing environmental conditions, if we want to understand how diazotrophy is contributing to the functioning of reef ecosystems. However, because of the uncertainty in (i) the distribution of N_2 fixation in coral reefs and (ii) the tightness of the coupling between N_2 fixation and primary production, the ecological significance of N_2 fixation in coral reef ecosystems is still not resolved. Thus, the objective of this paper is to provide an assessment of the quantitative importance of the process of N_2 fixation for primary production in coral reef ecosystems. While some studies attempted to do this before (Capone and Carpenter 1982; Capone 1996), no systematic and comprehensive analyses considering the multifarious diversity of coral reef substrates have been performed so far that would allow for a synthesis. Using results from a year-long study at a highly seasonal fringing reef in the northern Red sea, we evaluate here the contribution of all key benthic players to the production of fixed C and N in the reef environment. Moreover, geographic information system (GIS) analyses allow us to discuss the relative importance of all typical reef-associated habitats to primary production and N_2 fixation of the entire ecosystem. Finally, we indicate

the most probable environmental factors driving N_2 fixation in coral reef habitats, with consequent implications for predicting the potential effects of climate change on this important biogeochemical process.

Materials and methods

Study site

This study was carried out at a northern Red Sea fringing reef (Aqaba, Jordan) during two expeditions (January - April 2013) and (August - December 2013). Four sampling campaigns, each encompassing three weeks, were performed in February, April, September, and November, respectively. The timing of these periods (hereinafter called winter, spring, summer, and autumn respectively) was determined from literature to best cover the annual diversity in environmental conditions in this area of the Red Sea, characterized by an annual cycle of deep water mixing from December until May and stratification down to 200 m water depth from June until November (Silverman *et al.* 2007; Carlson *et al.* 2014). All measurements were conducted on site in the aquarium and laboratory facilities of the Marine Science Station (MSS, University of Jordan; location: 29° 27′ N, 34° 58′ E) and monitoring and sample collection took place on the adjacent fore reef slope at 10 m water depth. The fringing reef system extends approximately 1.1 km along its reef crest bordering the coastline. This reef has the typical morphology and zonation of Red sea fringing reefs, and can be partitioned into a reef flat, a reef crest and slope (hereafter: reef crest), and a forereef facing the open sea (Mergner and Schuhmacher 1974; Naumann *et al.* 2012). The fore reef consists of an upper, middle and lower part characterized by distinctive morphological features and species composition (hereafter: sand belt, transition zone and forereef). The present study focuses on the reef area framed by the reef flat and the fore reef slope at the maximum investigated depth (depth range: 0.5 - 20 m).

Environmental monitoring

Monitoring of the key environmental parameters was carried out as described in van Hoytema *et al.* (Chapter 3). Briefly, light availability (lux) and water temperature were measured at 1 min frequency with Onset HOBO data loggers (Pendant UA-002-64; temperature accuracy: ± 0.53 °C, spectral detection range: 150 – 1200 nm), and lux measurements were subsequently converted to photosynthetically active radiation (PAR) using a conversion factor obtained from parallel measurements with a LI-COR LI192SA underwater quantum sensor. Sampling for dissolved inorganic nitrogen (DIN = NH_4^+ + NO_x), dissolved inorganic phosphorous (DIP), chlorophyll *a* (Chl *a*), dissolved organic carbon (DOC), particulate organic carbon (POC) and particulate nitrogen (PN) was carried out once a week by SCUBA using high density poly-ethylene (HDPE) containers. Subsamples for inorganic nutrients (50 mL) were filtered through cellulose acetate filters (nominal pore size = 0.45 µm), and nutrient concentrations were measured according to Holmes *et al.* (1999), Murphy and Riley (1962), and Strickland and Parsons (1972). NH_4^+ was measured with a Trilogy

Fluorometer (Turner Designs), while PO_4^{3-} and NO_x were measured with a JASCO-V630 photometer. Detection limits for NH_4^+, PO_4^{3-}, and NO_x were 0.09, 0.01, and 0.02 µmol L^{-1}, respectively. Subsamples for Chl *a* (1 L) were filtered onto pre-combusted GF/F filters and stored in the dark at -80 °C. Chl *a* was later extracted with 90 % acetone for 12 h in the dark at 4 °C and measured on the Trilogy Fluorometer using the non-acidification module (CHL NA #046, Turner Designs). Subsamples for DOC (50 mL) were vacuum filtered (max. pressure 20kPa) through a pre-combusted GF/F filter into 30 mL HDPE bottles using an acid-washed and MQ and sample-rinsed filtration apparatus, acidified with 80 µL of 18.5 % HCl and stored at 4 °C in the dark until analysis. Samples were measured using the high-temperature catalytic oxidation (HTCO) method on a Shimadzu TOC-V_{CPH} TOC analyser with analytical precision of < 3 %. Subsamples for POC (1 L) and PN (2 L) were filtered onto pre-combusted GF/F filters (nominal pore size = 0.7 µm), which were subsequently dried at 40 °C for 48 h and sealed in Eppendorf tubes pending further analysis. Prior to their measurement, POC filters were acidified with 0.1 N HCl to remove any inorganic carbon. POC and PN samples were measured using a EuroVector elemental analyser (EURO EA 3000) with an analytical precision of ≤ 0.1 % (C) and ≤ 0.03 % (N).

Benthic community composition

Line point intercept surveys (LPI) were conducted to quantify benthic community composition and identify the dominant benthic categories. In each season, three LPI, each of 50 m length, were carried out at 0.5, 1.0, 5.0, 10.0 and 20.0 m water depth, at increasing distance from land along transects perpendicular to the shore-line, for a total of 120 LPI. The transect depths were selected as in previous studies (Naumann *et al.* 2012) to include all major reef habitats and to be representative of the seafloor coverage by all dominant benthic categories. Intervals of 0.5 m between intercepts resulted in 101 data points per LPI. Results obtained from all LPI in each depth were used to calculate the percentage coverage for each reef habitat (i.e., reef flat, reef crest, sand belt, transition zone and forereef) in each season (Appendix VII - Table 1), and to select the benthic categories that together accounted for ca. 90% of the 2D reef seafloor coverage on average (Appendix VII - Table 2) to be subsequently incubated.

Reef bathymetry and zonation

Bathymetric contours were obtained merging data from the Aqaba Special Economic Zone Authority (ASEZA) GIS Unit and original bathymetric data obtained in the field, overlaid onto a high-resolution satellite image (© Digital Globe). Bathymetric contours were subsequently processed into a continuous raster using the ArcGis Topo to Raster tool (ESRI ArcGis®), with the final raster having a cell size of ~ 2.9 m. The habitat map was produced based on prior knowledge of the reef zonation (Mergner and Schuhmacher 1974), on the interpretation of the high-resolution satellite image, and on ground truthing during the line point intercept surveys. Finally, zonal statistics referring to the bathymetric raster was calculated for each habitat using ArcGis 3D Analyst tools. The slope 2D area (i.e., considering the depth profile) for each habitat was obtained from the planar 2D area using the

bathymetric raster as base layer. The bathymetric and habitat maps are presented in Fig. 10.1, and zonal statistics for each habitat are reported in Appendix VII - Table 2 and were used in our subsequent ecological analyses.

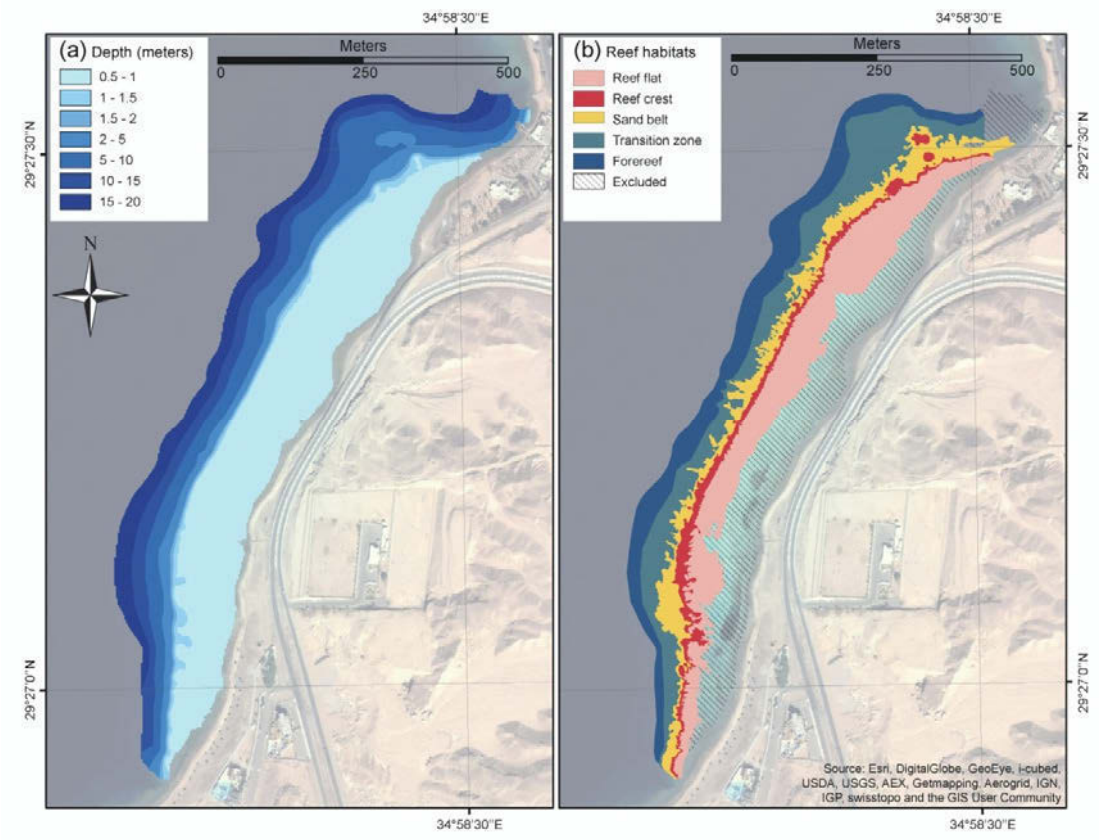

Figure 10.1. Bathymetry (a) and habitat (b) maps of the study site.

Sample collection and surface area quantification

In each season, specimens of the key benthic categories were collected for measurement of primary production and dinitrogen fixation. For an overview and description of the benthic categories incubated and respective replication see Appendix VII - Table 3. All benthic categories were sampled on the reef and transported back to the MSS facilities within 60 min of sampling. Hard and soft corals, as well as turf algae covered branches, were fixed to ceramic tiles with Reef Construct. Coral rock fragments were stable without fixation. Macroalgae were fixed to ceramic tiles or Petri dishes with rubber bands while protecting the algal tissue from crushing. Sediment and cyanobacterial mat samples were collected with round PVC sediment corers (internal diameter 43 mm) and transported back to the MSS laboratory where the top 1 cm of the core was extruded and placed in a Petri dish with the same internal diameter as the sediment corer. Organisms were maintained in an outside flow-through aquarium of 800 L with an exchange rate of 4000 L h^{-1} of water which was pumped straight from the coral reef at 10 m water depth, thus resembling in situ conditions of temperature and inorganic nutrients. In situ light conditions from 10 m water depth were generated using layers of

black mesh and monitored with data loggers (Onset HOBO Pendant UA-002-64). Hard and soft corals were maintained for at least 7 d, and macroalgae for at least 24 h, to recover from extraction. Sediment, turf algae, coral rock, and cyanobacterial mats were incubated on the day of collection to minimize any changes in their communities before incubation. The 3D surface areas of all benthic categories was also quantified. 3D surface areas of hard and soft corals, turf algae, and coral rock were measured using advanced geometry (Naumann *et al.* 2009; Bednarz *et al.* 2012). Macroalgae surface areas were measured by planar photograph analysis in ImageJ v.1.48 (Schneider *et al.* 2012), doubling the one-sided surface area to obtain the total 3D specimen surface area (Haas *et al.* 2010). Sediment and cyanobacterial mat surface areas were calculated from the circular surface area sampled by the sediment corer (πr^2). Specimen 3D surface area was related to the respective reef slope 2D area at the water-bottom interface determined during LPI surveys to generate the 2D to benthos 3D area conversion factor for each taxon. The specific resulting 2D to 3D conversion factors are summarised in Appendix VII - Table 3.

Physiological measurements

Specimens were incubated for measurements of net photosynthesis, dark respiration and dinitrogen fixation. Briefly, incubations were conducted under *in situ* conditions in the flow-through aquarium in closed glass chambers (0.5-1 L). Magnetic stirrer plates (Cimarec™ i Telesystem Multipoint Stirrers, Thermo Scientific™) and Teflon-coated stir bars in the chambers provided water movement during the incubations at 600 rpm. All material used to fix organisms was thoroughly cleaned before each incubation with a fine brush to remove potential epi-biota. Net photosynthesis and dark respiration of each specimen were quantified by their respective oxygen (O_2) fluxes in the light (i.e., net photosynthesis) and in the dark (i.e., dark respiration) with an O_2 optode and a conductivity probe (MultiLine® IDS 3430, WTW GmbH, Weilheim, Germany, accuracy: ± 0.5% of measured value). In addition, seawater controls with and without fixation tiles were run in parallel to correct for metabolic activity in the incubation seawater or potential microbiota on the tiles. Specimen dinitrogen fixation was quantified using the acetylene (C_2H_2) reduction assay during a full dark-light cycle incubation (20 hours), replacing 10% of the water volume with C_2H_2-enriched seawater and 10% of the headspace with C_2H_2 gas, both produced using calcium carbide. Gas samples were collected from each chamber at time intervals (0, 4, 12, 16 and 24 h) and transferred into gastight 2 ml vials previously filled with distilled water, subsequently stored frozen upside down until analysis. Ethylene (C_2H_4) concentrations were measured using a customized reducing compound photometer (RCP) (Peak Laboratories, detection limit = 100 ppb), calibrated with serial dilutions of a 200 ± 4 ppm C_2H_4 standard in air (Restek, Bellefonte, PA, USA). In addition, 4 sets of controls were also tested for ethylene (C_2H_4) production: 1. unfiltered seawater control (without fragments, n = 8); 2. 0.2 µm-filtered seawater control (without fragments, n = 6); 3. tiles in unfiltered seawater (without fragments, n = 6); 4. fragments in unfiltered seawater without C_2H_2 addition (natural C_2H_4 production, n = 6). Only unfiltered seawater showed measurable ethylene (C_2H_4) production. C_2H_4 fluxes were calculated following the endpoint assays equations of Capone (1993). Finally, O_2 and C_2H_4 fluxes of the

biological samples were corrected for unfiltered seawater control signals and normalized to incubation time and specimen 3D surface area.

Data treatment

O_2 and C_2H_4 fluxes were converted into carbon (C) and nitrogen (N) fluxes assuming theoretical molar ratios of CO_2:O_2 = 1 and C_2H_4:N_2 = 4. Whilst we acknowledge that these ratios may vary depending on the biological system under study (Larkum 1988; Larkum *et al.* 1988; Falkowski and Raven 2007), many systems display ratios not substantially different from these theoretical ratios (Capone 1988; Clavier *et al.* 1994), and the use of the conservative theoretical ratios does allow here to compare C and N_2 fixation rates by different reef organisms. To estimate daily gross primary production (GPP), every respiration measurement was added to its corresponding net photosynthesis measurement and extrapolated to per day estimates assuming 12 hours of daylight, while daily respiration (R) and dinitrogen fixation (BNF) were calculated on a 24 hours diel cycle. The daily net primary production (NPP) was obtained by subtracting each GPP measurement by its respective R. Daily C and N fluxes obtained were then scaled to the reef benthos 3D area considering the 2D:3D factors calculated for each benthic category (i.e., taking into account their respective three-dimensional structure) and are reported in Appendix VII - Table 4 expressed as mmol (C or N) m^{-2} benthos 3D area d^{-1}. To extrapolate our data to the ecosystem level, individual rates were used to calculate the contribution of each investigated benthic category to benthic GPP, NPP and BNF taking into account the specific percentage cover related to the slope 2D area of each reef habitat in each season (Appendix VII - Table 2). Here, rates measured under light conditions resembling 10 m water depth were extrapolated to different reef habitats (i.e., depths, range = 0.5 - 20 m). While this assumption may underestimate or overestimate the relative contribution of each reef habitat to the whole reef area, we believe it does provide a solid basis for assessing the overall significance and range of C and N_2 fixation that may occur at the ecosystem level. To allow comparison with other systems, resulting contributions in mmol (C or N) d^{-1} were subsequently normalised to the planar 2D reef area and are expressed as mmol (C or N) m^{-2} planar 2D area d^{-1}. To estimate the percentage contribution of BNF to NPP, a C:N:P ratio of 550:30:1 was assumed for reef benthic autotrophs (Atkinson and Smith 1983; Atkinson and Falter 2003).

Statistical analysis

Analyses were conducted using Primer-E v6 (Clarke and Gorley 2006) with the PERMANOVA extension (Anderson *et al.* 2008). Differences in the benthic community cover among seasons (fixed, four levels) and reef habitats (fixed, five levels) were tested for significance using multivariate analysis of variance (PERMANOVA) on a Bray-Curtis similarity matrix of square root transformed data. Type I (sequential) sum of squares was used with permutation of residuals under a reduced model (9999 permutations). Differences among habitats were visualised using principal coordinate analysis (PCO). Vector plots using Pearson ranking based on correlations > 0.4 were overlaid on the PCO plot to visualise relationships between variables and the ordination axes. Canonical analysis of principal

coordinates, CAP (Anderson *et al.* 2008) was used to identify specific groups in the benthic community responsible for differences among seasons, and benthic categories with correlations > 0.4 were overlaid on the plot as vectors. Differences in BNF, GPP, and R among seasons (four levels) were tested for significance using PERMANOVA on a Bray-Curtis similarity matrix of square root transformed data. Type I (sequential) sum of squares was used with unrestricted permutation of raw data (9999 permutations). If data did not conform to assumptions of homogeneity after transformation, tested with PERMDISP (Anderson *et al.* 2008), a conservative α value of 0.01 was used (Underwood 1997). Differences among seasons were visualized using PCOs, with benthic categories correlation vectors overlaid on the plots.

Results

Seasonal environmental conditions

Table 10.1. Environmental conditions during the four different seasons at the study site, and comparison with typical coral reef values as reported by Atkinson and Falter (2003) and Kleypas *et al.* (1999). Planktonic N_2 fixation rates are compared with rates obtained by Großkopf *et al.* (2012) for oligotrophic ocean waters using the ^{15}N dissolution method. Values are means with SD or range of values in parentheses.

Parameter	Winter	Spring	Summer	Autumn	Typical conditions
Temperature (°C)	22.3 (0.1)	22.4 (0.1)	27.0 (0.2)	24.9 (0.1)	27.6 (1.1)
Light (mol quanta m^{-2} d^{-1})	3.45 (0.26)	5.76 (0.13)	6.99 (0.32)	3.80 (0.34)	-
DIN (µM)	1.25 (0.13)	1.11 (0.07)	0.31 (0.06)	0.47 (0.09)	(0.1-1.1)
DIP (µM)	0.11 (0.01)	0.10 (0.01)	0.04 (0.01)	0.04 (0.01)	(0.05-0.3)
Chl *a* (µg L^{-1})	0.21 (0.01)	0.20 (0.02)	0.10 (0.00)	0.19 (0.02)	-
DOC (µM)	76.62 (3.82)	73.07 (3.13)	88.60 (1.93)	85.15 (1.46)	~100
POC (µM)	5.90 (0.56)	9.47 (1.39)	7.62 (1.01)	8.86 (0.38)	~10
PN (µM)	0.94 (0.05)	1.18 (0.14)	0.92 (0.11)	0.90 (0.04)	<1.0
POC:PN	6.81 (0.59)	7.95 (0.47)	8.25 (0.34)	9.86 (0.45)	~10
BNF (nmol N L^{-1} d^{-1})	3.1 (4.8)	3.3 (5.6)	8.1 (9.2)	2.6 (4.0)	2.5 (0.0-12.4)

Mean weekly measurements of environmental parameters were variable over time (Table 10.1) reflecting differences between the mixed and stratified seasons. Seawater temperature was low and stable throughout winter and spring but increased to 27 °C in summer and decreased again in autumn. Daily light availability increased from winter to spring and summer, while decreased in autumn. Inorganic nutrient concentrations and Chl *a* were significantly lower in summer and autumn than in winter and spring. Conversely, DOC showed a reversed pattern, with higher concentrations in summer and autumn. POC concentrations did not differ between the seasons, while PN was lower in summer and autumn, with subsequent increase of the POC:PN ratio. Planktonic N_2 fixation was

highly variable within seasons (Table 10.1), with no significant seasonal differences despite an average increase in summer. Overall, the stratified season resembled warm and highly oligotrophic conditions, typical of tropical coral reefs worldwide, while the mixed season had environmental conditions which were typical of marginal reef communities (Table 10.1, (Kleypas *et al.* 1999).

Benthic community composition

Analysis of LPI data yielded the percentage coverage by all benthic categories as reported in Appendix VII - Table 1. Multivariate analysis of the benthic community (Appendix VII - Table 5) showed a significant effect of the factors 'Season' and 'Reef Habitat' (PERMANOVA: P<0.001), but not of their interaction (PERMANOVA: P>0.1).

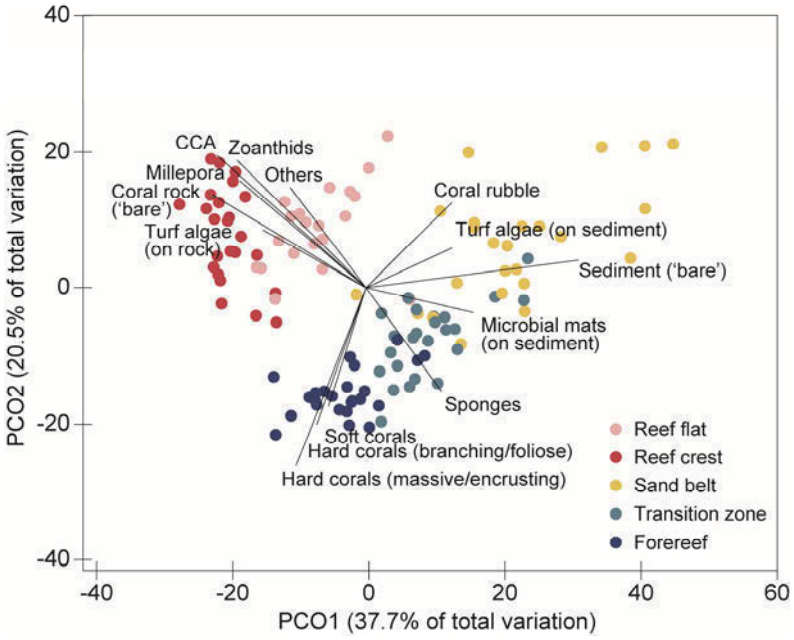

Figure 10.2. Principle coordinate analysis (PCO) of benthic community cover at the different reef habitats. Different colours represent different habitats as in the habitat map in Fig. 10.1. Vector overlay represent correlations > 0.4 based on Pearson ranking.

The different reef habitats are characterized by distinctive communities (Fig. 10.2). The reef flat was correlated with coral rock, turf algae on rock, crustose coralline algae (CCA) and zoanthids. The reef crest was highly correlated with coral rock, Millepora, turf algae on rock and CCA. Conversely, the sand belt was correlated with bare sediment, coral rubble and turf algae on sediment, whereas the fore reef was correlated with a high hard and soft coral coverage. The transition zone had an intermediate community between the sand belt and the fore reef, correlated with the presence of sponges, sediment and microbial mats on sediment, but also hard and soft corals (Fig. 10.2). Differences among seasons were less noticeable in the cloud of data, but the CAP analysis showed that seasonal changes were mainly attributable to macroalgae, microbial mats and turf algae cover (Appendix VII - Fig. 1), which

were higher in winter and spring (turf algae); only in spring (macroalgae and microbial mats on rock) or in spring and summer (microbial mats on sediment).

Metabolic rates of benthic primary producers

Benthic community metabolic rates showed significant differences between seasons (PERMANOVA: $P<0.001$ and pair-wise comparisons, Appendix VII - Table 6). GPP displayed a weak seasonal pattern, but generally increased in spring and summer compared to the other seasons for all benthic primary producers (Appendix VII - Fig. 2). Hard corals, soft corals, microbial mats and turf algae on rock displayed the highest rates of benthos 3D area-related primary production, while sediment the lowest (Appendix VII - Table 4). Respiration increased in summer for all benthic categories but for macroalgae and sediment that displayed higher respiration in spring compared to the other seasons (Appendix VII - Fig. 2). The highest benthos 3D area-related respiration rates were associated with sponges and hard and soft corals, the lowest with sediment (Appendix VII - Table 4). N_2 fixation rates associated with benthic primary producers showed the clearest seasonal pattern, with highest rates recorded in summer compared to all other seasons, except for sand-associated categories (macroalgae, sediment and microbial mats) that showed the highest associated N_2 fixation activity in spring compared to the other seasons (Appendix VII - Fig. 2). The highest benthos 3D area-related N_2 fixation rates were associated with microbial mats on rock, the lowest with soft corals and sponges (Appendix VII - Table 4).

Contribution of the benthic community to fixed C and N inputs

Scleractinian hard corals (all growth forms) contributed the largest share of benthic GPP (41 - 76 %) in all reef habitats and in all seasons (Fig. 10.3a). Their contribution to GPP was unrivalled even in the sand belt and the reef flat, where their benthic cover was generally less than 20 % (Appendix VII - Table 1). Soft corals importantly contributed to GPP in the forereef (8 - 11 %), in the transition zone (13 - 20 %) and on the reef flat (7 - 16 %) but their contribution was low in the other reef habitats. The contribution of 'bare' sediment to GPP was only relevant in the sand belt (4 - 20 %). 'Bare' hard substrates contributed importantly to GPP in the shallow reef habitats (i.e., sand belt, reef crest and reef flat) in winter, summer and autumn (11 - 26 %) but their contribution decreased significantly in spring (5 - 8 %) as a result of lower rates (Appendix VII - Table 4) and lower benthic coverage (Appendix VII - Table 1). Turf algae provided a large share of benthic GPP in shallow reef habitats (i.e., reef flat, reef crest) in winter and spring (9 - 20 %) but less in summer and autumn. Turf algae also importantly contributed in winter in the sand belt (11 %) because of a sharp increase in their benthic coverage (Appendix VII - Table 1, turf algae on sediment). Macroalgae were less important to the overall reef GPP, but their contribution increased in shallow and sandy reef habitats (i.e., reef flat, reef crest, sand belt) in spring (6 - 10 %). Microbial mats contributed only minor fractions to reef GPP, but their contribution was highly variable and depended on their benthic coverage, increasing up to 11 % on the reef flat in summer (Fig. 10.3a). Conversely, sponges showed negligible contributions to benthic GPP in all reef habitats and in all seasons. Benthic categories mostly

contributing to BNF (Fig. 10.3b) were microbial mats, turf algae, 'bare' hard substrates and 'bare' sediment, thus drawing a very different picture if compared with the categories contributing most to GPP (Fig. 10.3a). Despite very low benthic coverage (0 - 1.7 %) microbial mats provided a large fraction of BNF in spring in all reef habitats (27 - 64 %), but their contribution to BNF was highly variable in the other seasons. Turf algae contributed the largest share of BNF in winter in all reef habitats (41 - 66 %), but were also important in the other seasons, particularly in the reef crest and reef flat (12 - 41 %). 'Bare' hard substrates importantly contributed to BNF in all reef habitats and seasons (11 - 69 %), but particularly in summer and autumn in the reef crest and reef flat (38 - 69 %). 'Bare' sediment contributed a large fraction of BNF in the sand belt in all seasons (31 - 41 %), and was also important for BNF in the transition zone (17 - 34 %). The contribution of hard corals was low but occasionally relevant particularly in the forereef, increasing up to 18 % in winter and 17 % in summer. Macroalgae showed significant individual BNF rates but very low benthic coverage (Appendix VII - Table 1, 4), consequently contributing a low fraction of BNF to the reef (Fig. 10.3b). Sponges and soft corals showed negligible contributions to BNF in all habitats in all seasons (Fig. 10.3b).

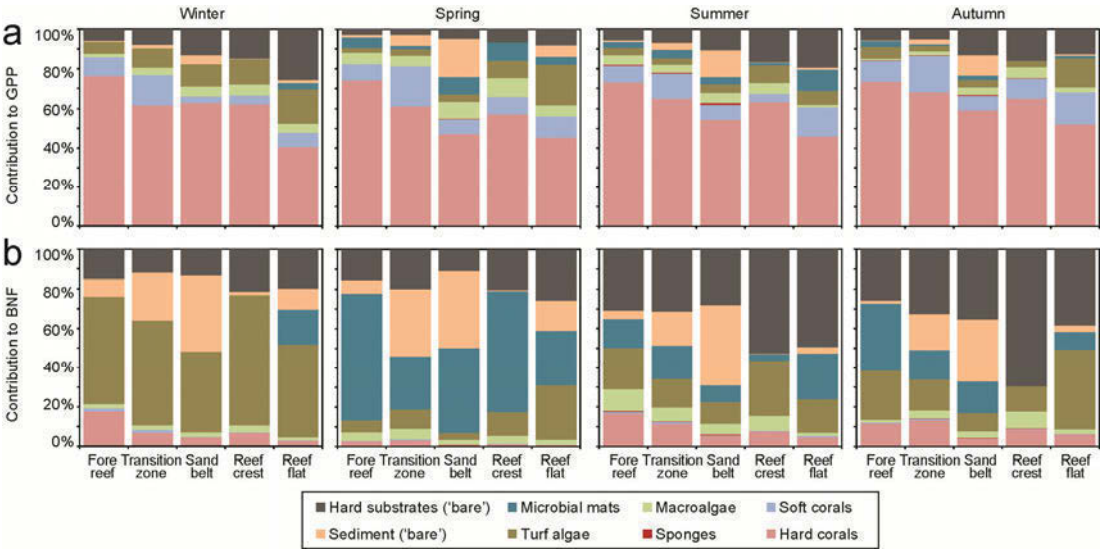

Figure 10.3. Contribution of the main benthic categories to GPP (a) and BNF (b) during the four seasons, in each reef habitat. All hard coral morphologies are classified as one category; coral rock and dead corals are grouped into hard substrates ('bare'); macroalgae, microbial mats and turf algae include categories 'on rock' and 'on sediment'.

Productivity and dinitrogen fixation in the different reef habitats

Overall, the contribution of the water column to GPP and BNF to the entire reef system was negligible (Fig. 10.4a, b). Total areal GPP increased in spring and summer compared to the other seasons, but remained high all year around (Fig. 10.4a). Reef habitats that showed the highest areal rates were the ones with the highest hard coral cover (i.e., reef crest, transition zone and forereef). The

lowest GPP was associated with the sand belt in all seasons. GPP was highest in summer in shallow reef habitats (i.e., reef crest and reef flat), while was highest in spring in the other reef habitats.

Table 10.2. Community dinitrogen fixation (BNF), gross primary production (GPP), respiration (R), net primary production (NPP) in mmol (C or N) m^{-2} planar 2D area d^{-1} and % contribution of BNF to NPP in the different reef habitats. Values are means followed by the range in parentheses.

Habitat	BNF	GPP	R	NPP	% Contribution
Water column	0.03 (0.02-0.06)	5 (3-7)	46 (30-55)	-	-
Reef flat	0.62 (0.22-1.18)	205 (178-240)	109 (86-145)	96 (88-112)	11.7 (4.4-22.9)
Reef crest	0.61 (0.15-1.07)	246 (222-266)	137 (121-165)	109 (88-139)	10.0 (2.6-19.2)
Sand belt	0.49 (0.14-0.83)	149 (117-173)	85 (75-97)	65 (42-95)	13.7 (4.0-23.8)
Transition zone	0.41 (0.14-0.80)	268 (234-303)	154 (134-186)	114 (95-156)	6.7 (2.6-14.2)
Forereef	0.50 (0.10-0.78)	327 (284-359)	190 (167-223)	137 (116-187)	6.6 (1.6-12.0)

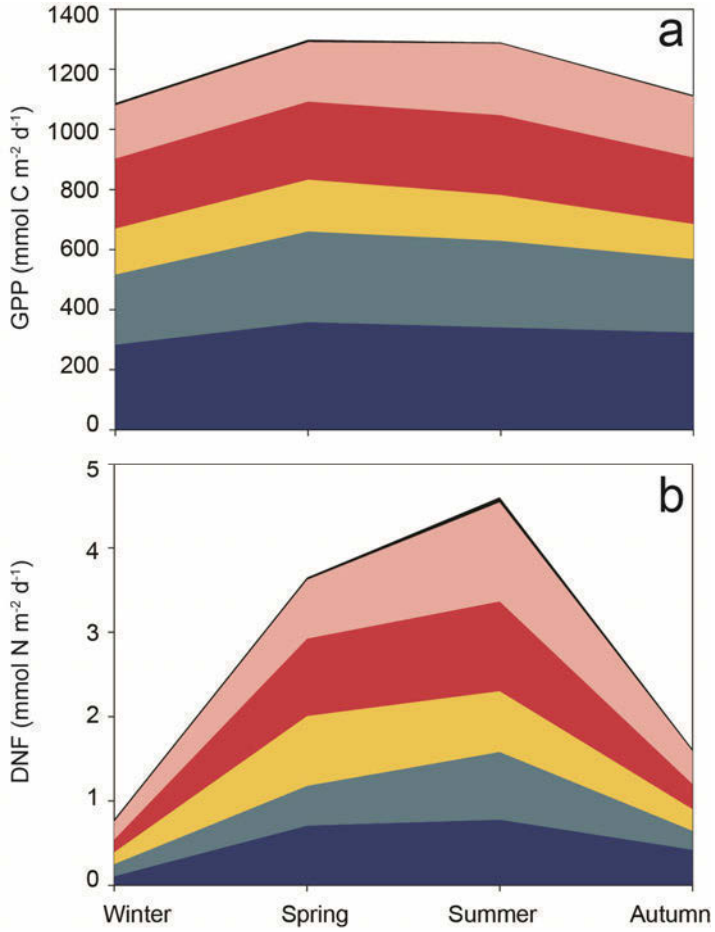

Figure 10.4. GPP (a) and BNF (b) in the different reef habitats in the different seasons. Different colours represent different habitats as in the habitat map in Fig. 10.1, and black represents the contribution of the water column.

Total areal BNF displayed a strong seasonal pattern with 4-fold higher rates in spring and summer compared to winter and autumn (Fig. 10.4b). The highest areal BNF rates were associated with the reef flat and the reef crest. BNF in the sand belt was highest in spring, while BNF in all the other reef habitats was highest in summer. On an annual average, the contribution of BNF to NPP was highest in the sand belt, and lowest in the forereef (Table 10.2).

Discussion

This study represents one of the very few field investigations succeeding early fundamental works (Wiebe *et al.* 1975; Larkum *et al.* 1988) that generates a dinitrogen fixation (BNF) budget for a coral reef community based on individual rates of all dominant benthic substrates. To our knowledge, it is the first to calculate a seasonal BNF budget by combining substrate-specific rates extrapolated to their respective benthic 3D surface areas with a GIS analysis of the reef area under study. Finally, this study is the first effort to constrain previous global estimates with an assessment of the associated uncertainty (Table 10.3), propagated according to the propagation of errors for linear combinations (Miller and Miller 2005). BNF rates for the individual substrates investigated (Appendix VII - Table 4) fall well within those reported by previous literature, when these values exist (see Cardini *et al.* (2014) for a review). At the global scale, no such detailed budget has been attempted previously, and values provided in Table 10.3 show that the estimates obtained here are reasonably robust against the associated uncertainties. However, there is considerable uncertainty in any such budget, and the increased complexity of the analyses introduces new uncertainties in our budget that include: (i) deviations from the theoretical C_2H_4:N_2 ratio in different communities due to, for example, the ratio of N incorporated into biomass versus the N released; (ii) spatial variations in activity associated with the same substrates caused by the effect of depth (i.e., light availability) on the measured processes; (iii) changes of ecosystem stoichiometry over space and time; (iv) changes in estimates of global coral reef areas. Nonetheless, some results appear robust:

– A clear decoupling exist between the substrates contributing most to GPP and the ones that contribute most to BNF (Fig. 10.3). This indicates that generally neglected benthic substrates such as turf algae, 'bare' hard substrates and sediments contribute indirectly to the overall benthic production by providing bioavailable N to the reef ecosystem (Wiebe *et al.* 1975; Shashar *et al.* 1994b; den Haan *et al.* 2014). Importantly, other reef organisms provide both ecosystem functions thanks to a symbiosis (e.g., hard corals) or to an inherent capability (e.g., microbial mats). These components are fundamental to both processes, with their contribution strongly depending on their benthic coverage.

- In coral reef ecosystems, BNF other than GPP are strictly benthos-related processes. The entire water column above the reef contributed with rates of BNF and GPP which were ca. 20-fold and 50-fold lower, respectively, than those contributed by the benthos (Table 10.2, Fig. 10.4).
- Regionally important reef-contiguous habitats (e.g., sandy lagoon areas) may be major contributors of new N into the reef ecosystem as previously indicated (Capone *et al.* 1992; Shashar *et al.* 1994b; O'Neil and Capone 2008). However, contribution by high-coral cover areas was overlooked. These contribute new N at rates similar to that of sandy areas when accounting for their three-dimensional surfaces (Fig. 10.4). Moreover, these habitats are unrivalled in terms of GPP, shaping coral reef ecosystem productivity and growth.
- BNF is highly susceptible to changes in environmental conditions, with 4-fold higher areal rates in spring and summer compared to winter and autumn in all reef habitats (Fig. 10.4). This suggests that BNF will likely respond to a greater extent than GPP to future climate change. Because of the tight coupling of BNF with the reef C cycle, with BNF sustaining ca. 10 % of NPP of the entire reef system on an annual basis, and up to ca. 20 % of NPP in summer (Table 10.2), BNF dynamics need to be accounted for when projecting the future of coral reef ecosystems productivity in response to climate change.

Concluding remarks

In an attempt to scale our results and provide estimates of global N inputs from benthic N_2 fixation in coral reef ecosystems, we used the global reef area provided by Burke *et al.* (2011) to quantify the contribution by the reef-framework (i.e., reef flat, reef crest, transition zone and fore reef), while the difference between the estimate by Smith (1978) and the one by Burke *et al.* (2011) was used to quantify the contribution by the reef-contiguous soft bottom communities (i.e., sand belt). In addition, the fringing reef under study is exposed to seasonally changing environmental conditions. The stratified summer resembles typical conditions of tropical reef communities worldwide, which are characterized by warm and highly oligotrophic waters, while the mixed season has similar conditions to the ones found in marginal reef communities where nutrient concentrations are higher and temperature and light intensity are lower. Consequently, we present here different estimates based on annual averages and on summer measurements only, as the latter ones may more closely approximate the scale and significance of N_2 fixation in tropical reef systems worldwide (Table 10.3). These estimates, particularly those based on summer measurements, are similar to those presented by Capone and Carpenter (1982). However, they based their calculations on early measurements of N_2 fixation in algal mats (Webb *et al.* 1975; Wiebe *et al.* 1975) which were one order of magnitude higher than those measured in subsequent studies in different locations but for similar substrates (Wilkinson

et al. 1984; Charpy-Roubaud *et al.* 2001; Charpy-Roubaud and Larkum 2005). In any case, our estimates approach that of Capone and Carpenter (1982) due to the meanwhile increase of global reef area estimates. Ranging from 1.60 to 2.58 Tg N y^{-1}, our extrapolation define coral reef ecosystems among the benthic communities contributing most to the inputs of fixed N on a global scale.

Table 10.3. Estimate of the total contribution of combined nitrogen to the global nitrogen cycle by nitrogen fixation in benthic coral reef environments, revised after Capone and Carpenter (1982) (with data from Wiebe *et al.* (1975)) and (Larkum *et al.* 1988). Values are means with SE or range of values in parentheses.

Study location	Community	Time of year	Areal rate (μmol N m^{-2} d^{-1})	Area (10^3 km^2)	N fixed (Tg N yr^{-1})	Reference
Aqaba	Reef framework	Annual average	536 (69)	249.7[a]	0.68 (0.09)	This study
Aqaba	Soft bottom	Annual average	488 (62)	367.3[a]	0.92 (0.12)	This study
Aqaba	Total reef areas	Annual average	-	617.0[a]	1.60 (0.15)	This study
Aqaba	Reef framework	Summer	956 (151)	249.7[a]	1.22 (0.19)	This study
Aqaba	Soft bottom	Summer	723 (110)	367.3[a]	1.36 (0.21)	This study
Aqaba	Total reef areas	Summer	-	617.0[a]	2.58 (0.28)	This study
Enewetak Atoll	Algal mats	Jan - Feb	4890 (1643)	110.0[b]	2.8	Capone and Carpenter (1982)
One Tree Reef	Various substrates	Annual range	(156-1330)	-	-	Larkum *et al.* (1988)

[a] The estimate from Burke *et al.* (2011) was used for reef framework areas and the estimate by Smith (1978) was used to calculate the areal extent of reef-associated soft bottom communities.

[b] The old estimate from De Vooys (1979) was used as in the original publication.

Areal GPP and BNF rates were highest in spring and summer: it follows that the environmental factor controlling most these two processes at the reef scale was light availability, which also increased in spring and summer (Table 10.1). Secondarily, temperature was also exerting an important control on BNF, as summer individual rates were the highest. This is consistent with previous studies that found BNF to be light and temperature dependent on coral reef substrates (Wilkinson *et al.* 1985; Larkum *et al.* 1988). Conversely, the high concentrations of dissolved inorganic nutrients did not determine a decrease of overall reef BNF in spring (Fig. 10.4). Thus, it is possible that future anthropogenic global warming will more strongly affect BNF in coral reef ecosystems, rather than human induced eutrophication of the coastal zones. As yet, large uncertainties remain in the potential responses, requiring further assessments. It is clear, however, that the tight coupling between BNF and reef primary productivity will determine feedback loops where perturbations in BNF will have repercussions in the C cycle, and vice versa.

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Conclusions and outlook

Key findings and significance

While covering approximately 0.1% of the world's ocean surface, coral reefs may be home to 25% of all known marine species (McAllister 1995; Spalding *et al.* 2001). They are among the most productive ecosystems on Earth (Crossland *et al.* 1991), and provide valuable ecosystem services to millions of people (Moberg and Folke 1999). However, coral reefs face an intensifying array of threats (Burke *et al.* 2011), including impacts from overfishing, coastal development, agricultural runoff, and shipping (Fig. C-1). Moreover, the global threat of climate change has begun to jeopardize coral reefs and is projected to intensify in the future (Pandolfi *et al.* 2011). Ocean warming is causing coral bleaching events to increase in frequency and severity over wide reef areas (Donner *et al.* 2005). At the same time, ocean acidification is slowing coral growth rates potentially reducing their ability to build physical reef structure (De'ath *et al.* 2009). Recent studies have also demonstrated how these global stressors can impact the diversity and function of coral reef-associated microbial assemblages (Rosenberg and Ben-Haim 2002; Witt *et al.* 2011; Morrow *et al.* 2014; Santos *et al.* 2014; Thompson *et al.* 2015) suggesting concomitant effects on microbe-mediated biogeochemical cycling.

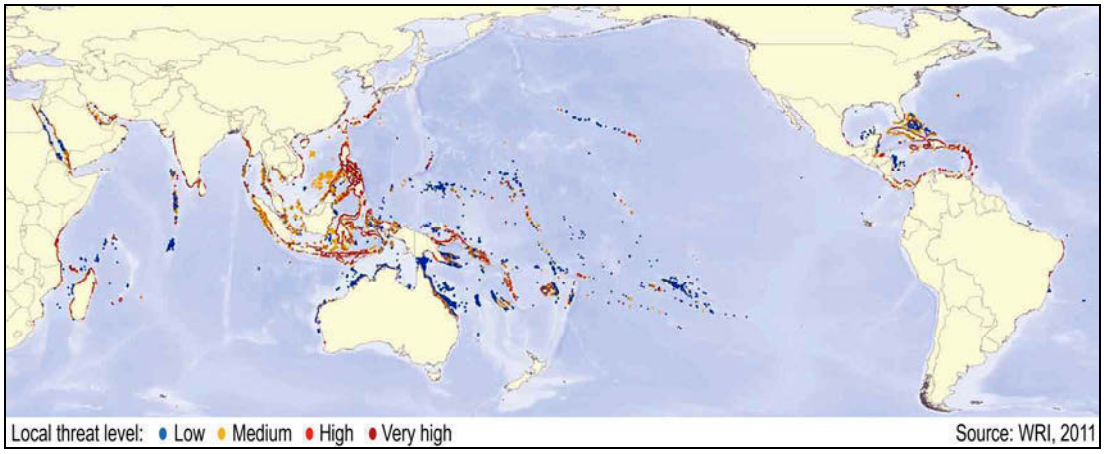

Figure C-1. Coral reefs classified by integrated local threat level (from Burke *et al.* 2011).

With this combination of local and global threats, coral reef ecosystems are becoming increasingly susceptible to disturbance or damage from storms and diseases, and there is a real risk that such degradation will result in decline of ecosystem productivity and functioning with consequent loss of associated ecosystem services (Hoegh-Guldberg *et al.* 2007). However, despite widespread recognition that coral reefs around the world are at serious risk, our knowledge regarding how coral reef ecosystems function is still limited, hampering conservation efforts. In this context, N_2 fixation is a fundamental biological process to consider if we are to understand the mechanisms of reef biogeochemical cycling. In particular, given the tight coupling between reef productivity and N_2 fixation, knowledge of large-scale distribution patterns and quantification of benthic diazotrophy is called upon in order to enable efficient management of coral reefs. The overall findings of the work

presented in this thesis provide the first comprehensive and comparative basis for future studies on benthic diazotrophy patterns and significance in other coral reef habitats. With reference to the three key objectives, the major and general conclusions of this thesis are:

1. Benthic N_2 fixation in tropical coral reefs is conspicuous and the rule rather than the exception in all benthic substrates investigated (Chapters 4 - 7), while the contribution of fixed nitrogen from the planktonic diazotrophic community is marginal (Chapter 3, 10). Rates of N_2 fixation vary greatly between the different substrates, indicating that ongoing changes in benthic community composition and/or structural habitat loss (Chapter 2) will result in changes of inputs of fixed nitrogen to the reef ecosystem. Furthermore, N_2 fixation rates are highly variable and dependant on environmental conditions, suggesting that disturbances resulting from human activities will also likely determine changes in the extent of N_2 fixation on reefs.

2. In this context, global stressors such as ocean warming and acidification have a high potential to influence N_2 fixation in scleractinian corals. Two scleractinian coral species showed similar and strong increases in light N_2 fixation rates when exposed to increased water temperature (Chapter 8). Thus, coral-associated diazotrophs show physiological plasticity under high temperature, fixing additional nitrogen that may play a beneficial role during thermal stress events. Conversely, increased pCO_2 caused a rapid and significant decrease in N_2 fixation activity in *Seriatopora hystrix* (Chapter 9). Changes in N_2 fixation activity were concurrent with alteration of other physiological processes such as photosynthesis and organic matter release (Chapter 8) or calcification (Chapter 9). While these processes are physically separated in the different members of the coral holobiont, they might compete for energy in corals exposed to climate change, causing cascading biogeochemical impacts.

3. While highest N_2 fixation rates were recorded for microbial mats and 'bare' consolidated and unconsolidated substrates, scleractinian corals also contribute significant amounts of fixed nitrogen when accounting for their structural complexity (Chapter 10). Thus, high coral-cover areas of the reef are not only important for their primary productivity but also for their associated N_2 fixation, with areal rates competing with those of flat sandy areas. N_2 fixation assures a constant supply of nitrogen to these often nutrient-deprived systems. In summer, when conditions at the study site resembled environmental conditions typically found in tropical reefs worldwide, N_2 fixation provided ca. 20% of the nitrogen needed for net primary production, highlighting the importance of this process for reef functioning. Finally, a global extrapolation of areal N_2 fixation rates places coral reef ecosystems among the benthic communities contributing most to marine fixed nitrogen inputs.

Future perspectives

Coral reefs exist within a narrow belt across the world's tropical oceans, where precise environmental and biological conditions combine to meet the exact requirements of reef-building corals (Burke *et al.* 2011). Studies investigating the effects of global warming and acidification on corals as the key ecosystem engineers have extensively demonstrated the negative effects of these stressors on their calcification and growth (see Wild *et al.* 2011 for a review). This thesis has demonstrated that N_2 fixation by symbiotic diazotrophs in corals will also be affected (Chapters 8, 9). If these processes compete for energy in the holobiont, a negative feedback loop may be triggered (Fig. C-2). Conversely, other benthic organisms such as fleshy and turf algae or microbial mats may be favoured in future climate change scenarios, as these often increase their productivity when exposed to higher temperatures and pCO_2 (see Gao *et al.* 2012 for a review). Microbial mats as well as macro and turf algae usually display high abundances of mat-forming diazotrophs and high rates of N_2 fixation (see Chapters 1, 6, 7, 10). Recent studies further demonstrated that warming and acidification promote cyanobacterial dominance in turf algal assemblages (Bender *et al.* 2014), and that macroalgal blooms can cause significant increases of diazotrophic abundance in the water column (Zhang *et al.* in press). Thus, changes in benthic community composition due to global (e.g. warming) and local (e.g. eutrophication) stressors may throw off balance the delicate equilibrium of the reef biogeochemical cycling, triggering profound modifications of the reef carbon and nitrogen cycle and ultimately causing shifts to different ecosystem states (e.g. algae and mats-dominated communities, Fig. C-2). Eventually, this may cause loss of ecosystem services that sustain millions of people living in tropical coastal zones.

Possibly, a successful equilibrium is even more fragile at the cellular level in the coral-algal-prokaryote symbiosis, where the interactions among the partners determine the overall fitness of the holobiont. Chapter 8 provides evidence that after a thermal stress event N_2 fixation activity increases in the dark, particularly in coral species that underwent bleaching. To explain these results, I propose here that *Symbiodinium* is the designated partner of diazotrophs in the coral host, and that the dinoflagellate algae are responsible for protecting diazotrophic activity from oxygen inhibition. In fact, given that nitrogenase is inactivated by oxygen, the process of N_2 fixation has to be spatially or temporally separated from photosynthesis (Berman-Frank *et al.* 2003). For this reason, in root nodules of the legume-rhizobium symbiosis, leguminous plants produce the oxygen carrier called leghaemoglobin, which is crucial for symbiotic N_2 fixation (Ott *et al.* 2005). This hemoprotein allows nitrogenase to function providing at the same time enough oxygen to the bacteria for their respiration (Ott *et al.* 2005). In corals, rhizobia dominate diazotrophic assemblages (Lema *et al.* 2012; Lema *et al.* 2014a; Lema *et al.* 2014b; Santos *et al.* 2014), and genes coding for similar hemoproteins were recently found within the transcriptome of *Symbiodinium* (Rosic *et al.* 2013). Moreover, expression of these genes

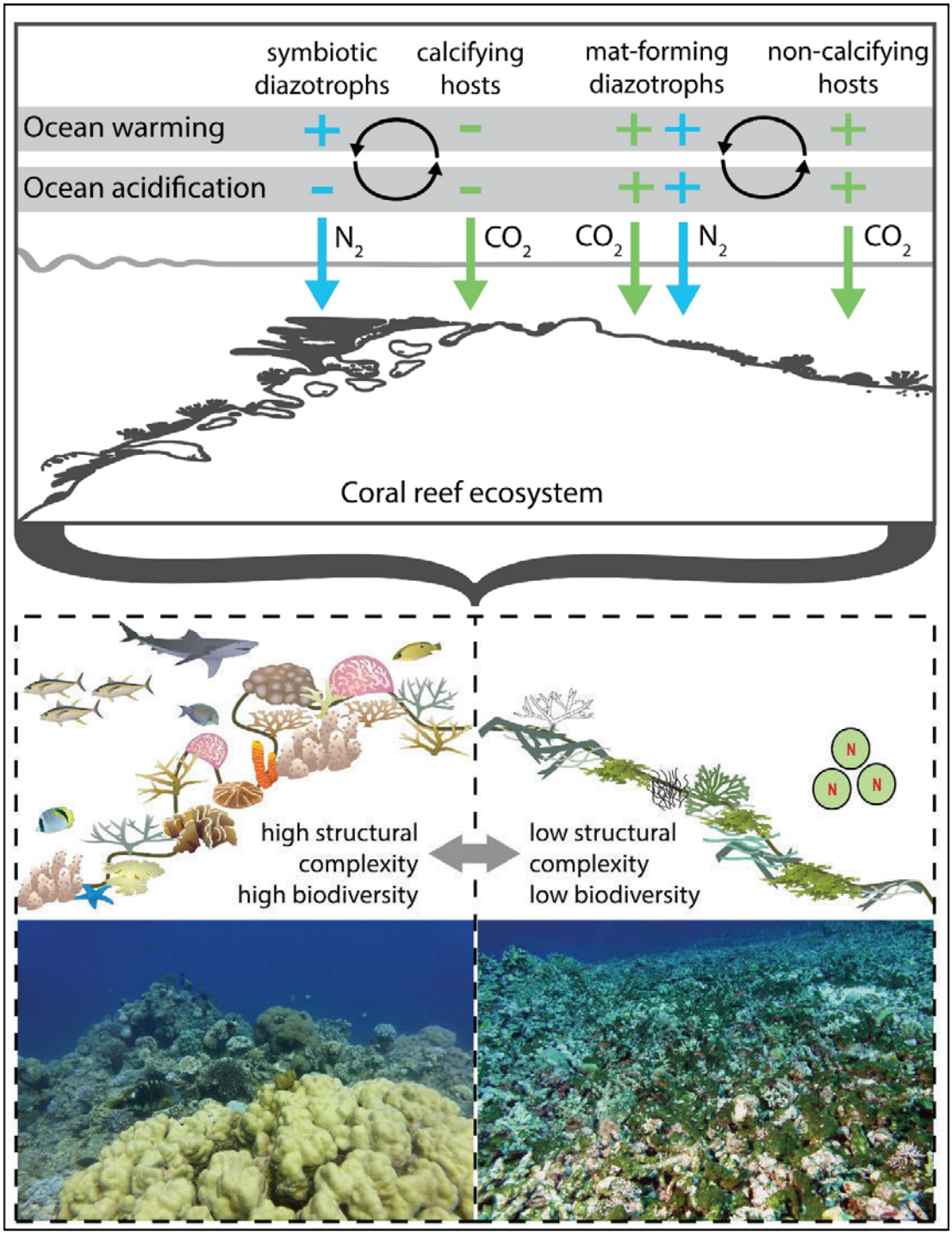

Figure C-2. Potential biogeochemical impacts of climate change on carbon and dinitrogen fixation as key reef ecosystem processes. In the upper panel, major groups of benthic diazotrophs as well as benthic autotrophs and potential trends in fluxes of nitrogen and carbon with ocean acidification and warming are shown. Please note that predictions of effects are highly tentative, and high variability in responses is present within the groups summarized here. In the lower panel, the equilibrium between two different reef ecosystem states.

increased with temperature and nitrogen concentrations (Rosic *et al.* 2013). In Chapter 8, the steady increase in light N_2 fixation activity under increased temperature was concomitant with sustained photosynthesis, thus requiring protection from oxygen. Conversely, when the coral-algal symbiosis was impaired and bleaching occurred, higher N_2 fixation was measured in the dark, concomitant with oxygen consumption by coral respiration. The capacity of *Symbiodinium* to produce a hemoprotein protecting nitrogenase from oxygen inhibition in the intact symbiosis could therefore explain these measurements. If the ability of *Symbiodinium* to produce such a protein is demonstrated experimentally, this would greatly inform our understanding of the evolution of symbiotic nitrogen fixation and of coral holobiont ecology.

Thus, many challenges remain in the field of exploring diazotrophy in coral reef ecosystems. One approach that can efficiently generate information on diazotrophs diversity in these valuable and vulnerable ecosystems is the use of environmental metagenomics. Concurrently, the use of transcriptomics allows the identification of genes that are differentially expressed in distinct communities, or in response to different treatments, while fluorescence in situ hybridization and secondary ion mass spectrometry open new possibilities for coupling phylogenetic identity and metabolic function in studies of N_2 fixing symbiotic systems. In this perspective, a multidisciplinary approach combining expertize from biogeochemistry, microbiology, molecular ecology, and physiology is necessary in future studies that seek to understand the intricate interconnections that result in a productive coral reef ecosystem and the many changes that these ecosystems are experiencing as a result of human activities.

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Appendices

Appendix I

Supplementary information Chapter 1

Appendix II

Supplementary information Chapter 2

Appendix III

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Appendix V

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Appendix VII

Supplementary information Chapter 10

Appendix I - Table 1. Values in this Table have been collected from the available literature and used to produce the graph in Fig. 1.3. Rates of N_2 fixation have been extrapolated from the text when possible, and the original conversion factor used by the authors has been reported. If no conversion was available, but only C_2H_2 reduction rates were reported, the conservative 4:1 conversion ratio has been used. ARA = Acetylene Reduction Assay, $^{15}N_2$ = labeling incubations with isotope $^{15}N_2$. Only studies with rates normalized to surface area are shown.

Substrate	Methods	Ratio (mol C_2H_2:1 mol N_2)	N_2 fixation rates (mg N m^{-2} day^{-1})	Location	References
Scleractinian corals	ARA	4.00	3.32 ± 3.70	Eilat, Israel	Shashar et al. (1994a)
	ARA	4.00	0.29 - 1.13	Great Barrier Reef, Australia	Davey et al. (2008)
	ARA	3.45	9.75 ± 8.18	Eilat, Israel	Shashar et al. (1994b)
	ARA	4.00	2.90 ± 2.19	Eilat, Israel	Shashar et al. (1994b)
	ARA	4.00	3.69 ± 1.60	Eilat, Israel	Shashar et al. (1994b)
	ARA	4.00	5.24 ± 0.25	Eilat, Israel	Shashar et al. (1994b)
	ARA	4.00	3.69 ± 4.96	Eilat, Israel	Shashar et al. (1994b)
	ARA	3.45	7.21 ± 2.69	Eilat, Israel	Shashar et al. (1994b)
	ARA	3.45	0.68 ± 0.45	Eilat, Israel	Shashar et al. (1994b)
	ARA	3.00	0.00 - 8.40	Akumal, Mexico	Lesser et al. (2007)
Other cnidarians	ARA	4.00	30.0 ± 11.93	Eilat, Israel	Shashar et al. (1994a)
	ARA	4.00	0.08 ± 0.25	Eilat, Israel	Shashar et al. (1994a)
Sponges	ARA	4.00	0.19 ± 0.34	Eilat, Israel	Shashar et al. (1994a)
Macro/Turf Algae	ARA	3.45	0.44 - 1.07	Great Barrier Reef, Australia	Larkum et al. (1988)
	ARA	3.45	8.18 ± 12.05	St. Croix, US Virgin Island	Williams and Carpenter (1997)
	ARA	4.00	22.69 ± 9.24	Kaneohe Bay, Oahu, Hawaii	Williams and Carpenter (1998)
	ARA	3.45	10.36 ± 0.56	Eilat, Israel	Shashar et al. (1994a)
Limestone/Dead corals	ARA	4.00	0.00 - 42.16	Great Barrier Reef, Australia	Wilkinson et al. (1984)
	ARA	4.00	0.51 ± 0.78	Eilat, Israel	Shashar et al. (1994a)
	ARA	3.45	10.72 ± 11.30	Great Barrier Reef, Australia	Larkum et al. (1988)
	ARA + $^{15}N_2$	3.30	2.12	Tuamotu Atoll, French Polynesia	Charpy-Roubaud et al. (2001)
	ARA	3.45	1.05 - 25.98	Great Barrier Reef, Australia	Davey et al. (2008)
	ARA	3.45	3.61 - 15.98	Great Barrier Reef, Australia	Larkum (1988)

	ARA	3.45	62.23 ± 31.94	Eilat, Israel	Shashar et al. (1994a)
	ARA	3.45	4.68 - 30.79	Great Barrier Reef, Australia	Larkum (1988)
Carbonate sands	ARA	3.00	0.06 - 0.26	Great Barrier Reef, Australia	Werner et al. (2008)
	ARA + $^{15}N_2$	3.00	1.80	Barbados	Patriquin and Knowles (1975)
	ARA	4.00	1.10 - 8.50	Great Barrier Reef, Australia	Wilkinson et al. (1984)
	ARA	3.00	0.30 - 2.40	Puerto Rico	Corredor and Capone (1985)
	ARA	4.00	0.13 - 4.08	Bermuda	O'Neil and Capone (1989)
	ARA	4.00	0.71 - 5.17	Puerto Rico	O'Neil and Capone (1989)
	ARA	4.00	0.07 - 0.69	San Salvador, Bahamas	O'Neil and Capone (1989)
	ARA	4.00	0.20 - 1.30	Great Barrier Reef, Australia	O'Neil and Capone (1989)
	ARA + $^{15}N_2$	3.16	2.00 - 10.00	Moreton Bay, Australia	O'Donohue et al. (1991)
	ARA	3.00	1.68 - 4.37	Great Barrier Reef, Australia	Capone et al. (1992)
	ARA + $^{15}N_2$	1.80 - 3.00	0.40 - 3.90	Tuamotu Atoll, French Polynesia	Charpy-Roubaud et al. (2001)
	ARA	3.45	21.87 ± 19.61	Eilat, Israel	Shashar et al. (1994a)
	ARA + $^{15}N_2$	3.45	0.34 ± 0.09	Great Barrier Reef, Australia	Larkum et al. (1988)
	ARA + $^{15}N_2$	3.45	0.81 ± 0.30	Great Barrier Reef, Australia	Larkum *et al.* (1988)
	ARA	4.00	8.21 ± 2.70	New Caledonia	Charpy et al. (2007)
Microbial mats	ARA	4.00	16.40 ± 5.40	New Caledonia	Charpy et al. (2007)
	ARA + $^{15}N_2$	1.60	8.00	Tuamotu Archipelago, French Polynesia	Charpy-Roubaud and Larkum (2005)
	ARA	4.00	110.00	Enewetak Atoll, Marshall Islands	Mague and Holm-Hansen (1975)
Seagrasses	ARA + $^{15}N_2$	3.16	19.00 - 40.00	Moreton Bay, Australia	O'Donohue et al. (1991)
	ARA + $^{15}N_2$	4.40	145.00	Great Barrier Reef, Australia	Iizumi and Yamamuro (2000)
	ARA	3.00	16.00 - 47.00	Gulf of Carpentaria, Australia	Moriarty and O'Donohue (1993)
	ARA	3.00	13.00 - 19.00	Gulf of Carpentaria, Australia	Moriarty and O'Donohue (1993)
	ARA	3.00	25.00	Gulf of Carpentaria, Australia	Moriarty and O'Donohue (1993)
	ARA	n.a.	28.00	Jamaica	Blackburn et al. (1994)
	ARA	n.a.	0.03	Florida, USA	McRoy et al. (1973)
	ARA	3.00	5.00 - 24.00	Biscayne Bay, Florida, USA	Capone and Taylor (1980)
	ARA	3.00	5.10 - 9.00	Bimini Harbour, Bahamas	Capone et al. (1979)
	ARA	3.00	27.00 - 140.00	Barbados	Patriquin and Knowles (1972)

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Appendix II - Table 1. List of benthic organisms numbered as they appear in Fig. 2.1. The systematics of the species is here reported as originally compiled by Mergner and Schuhmacher (1981).

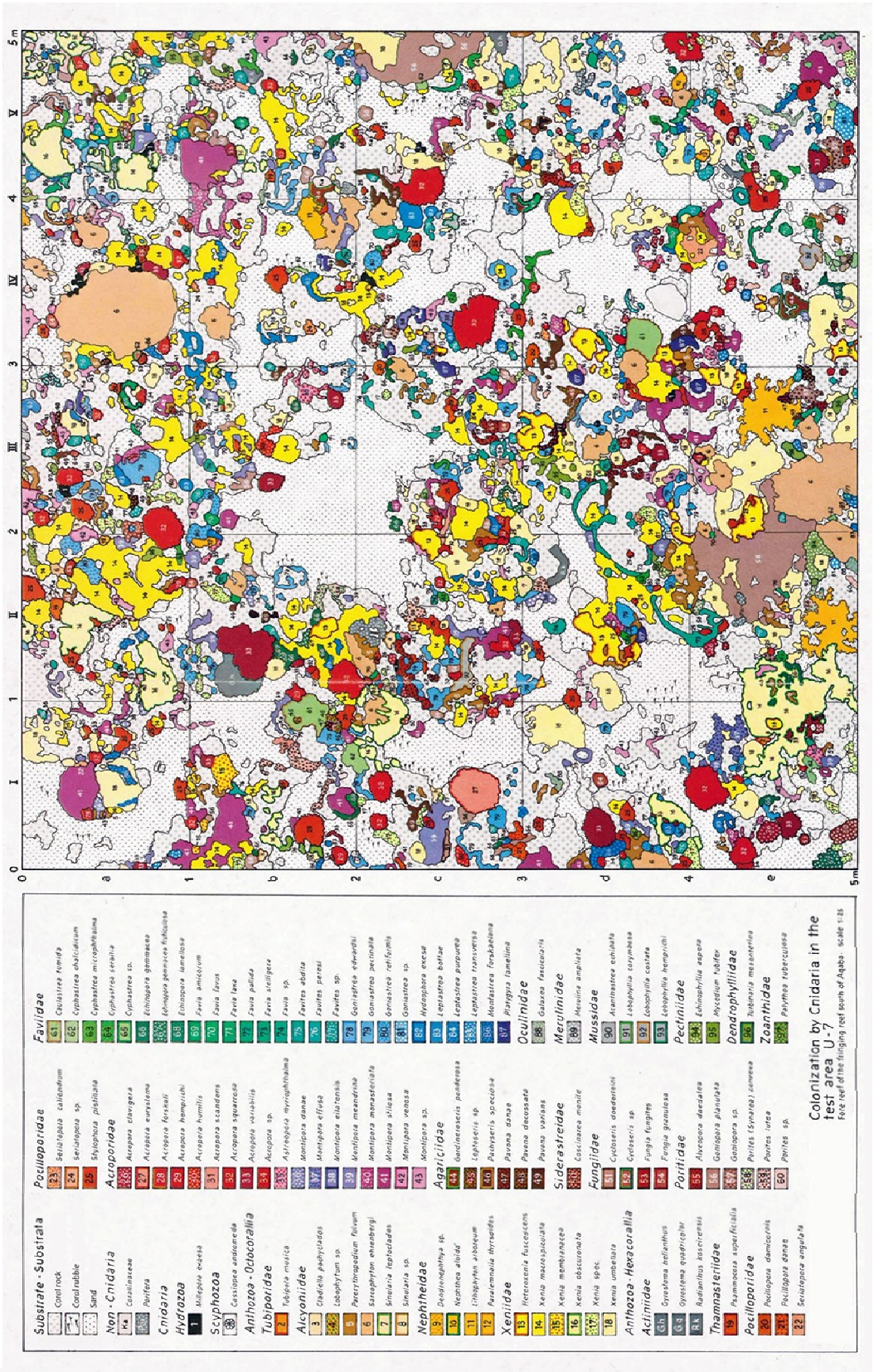
Nr.	Order, Genus, Species	Number of colonies	Contribution to total 5x5m area (%)	Contribution to total 5x5m area (cm²)	Average size of individual colony (cm²)	Size of respective largest colony (cm²)	Contribution to total live cnidarian cover (%)
	Hydroidea	16	0.09	241.94			0.23
1	*Millepora exaesa*	16	0.09	241.94	15.12	43.38	0.23
	Rhizostomeae	2	0.03	83.18			0.08
	Cassiopea andromeda	2	0.03	83.18	41.59	58.13	0.08
	Stolonifera	2	0.02	43.38			0.04
2	*Tubipora musica*	2	0.02	43.38	21.69	22.80	0.04
	Alcyonaria	749	20.62	51631.28			48.61
3	*Cladiella pachyclados*	12	0.14	393.89	28.32	54.11	0.32
4	*Lobophytum sp.*	1	0.01	25.04	25.04	25.04	0.02
5	*Parerythropodium fulvum*	92	1.02	2557.69	27.80	120.75	2.41
6	*Sarcophyton ehrenbergi*	28	3.26	8161.89	291.49	1451.69	7.69
7	*Sinularia leptoclados*	2	0.04	109.12	54.56	90.78	0.10
8	*Sinularia polydactyla + sp.*	2	0.03	63.95	31.97	44.72	0.06
9	*Dendronephthya sp.*	3	0.02	47.40	15.80	34.43	0.05
10	*Nephthea albida*	1	0.03	80.50	80.50	80.50	0.08
11	*Lithophyton arboreum*	3	0.80	1997.76	665.92	809.92	1.88
12	*Paralemnalia thyrsoides*	4	0.06	161.44	40.36	58.13	0.15
13	*Heteroxenia fuscescens*	29	0.93	2333.18	80.45	315.74	2.20
14	*Xenia macrospiculata*	232	5.70	14239.70	61.37	363.59	13.41
15	*Xenia membranacea*	8	0.13	330.94	41.36	239.26	0.31
16	*Xenia obscuronata*	61	2.59	6463.77	105.96	516.99	6.09
17	*Xenia spec.*	69	0.73	1832.73	26.56	98.38	1.73
18	*Xenia umbellata*	202	5.13	12832.28	63.52	1024.15	12.11
	Scleractinia	1274	21.49	53905.19			50.86
19	*Psammocora superficialis*	4	0.07	173.07	43.26	90.33	0.16
20	*Pocillopora damicornis*	3	0.05	127.90	31.97	68.42	0.12
21	*Pocillopora danae*	2	0.05	124.32	62.16	56.79	0.12
22	*Seriatopora angulata*	5	0.13	318.87	63.77	176.65	0.30
23	*Seriatopora caliendrum*	1	0.01	17.88	17.88	17.88	0.02
24	*Seriatopora sp.*	2	0.02	47.85	23.92	33.54	0.05
25	*Stylophora pistillata*	94	1.61	4031.30	42.88	169.94	3.80
26	*Acropora clavigera*	13	0.14	357.33	27.48	122.98	0.34
27	*Acropora eurystoma*	1	0.23	569.31	569.31	569.31	0.54
28	*Acropora forskali*	1	0.01	30.41	30.41	30.41	0.03
29	*Acropora hemprichi*	6	0.09	221.37	36.89	71.55	0.21
30	*Acropora humilis*	4	0.09	229.42	57.35	137.74	0.22
31	*Acropora scandens*	2	0.06	150.26	75.13	76.92	0.14
32	*Acropora squarrosa*	44	1.79	4474.50	101.69	614.93	4.22

Appendix II - Table 1. continued.

Nr.	Order, Genus, Species	Number of colonies	Contribution to total 5x5m area (%)	Contribution to total 5x5m area (cm²)	Average size of individual colony (cm²)	Size of respective largest colony (cm²)	Contribution to total live cnidarian cover (%)
33	*Acropora variabilis*	32	0.92	2287.56	71.48	345.10	2.15
34	*Acropora sp.*	10	0.06	145.34	14.53	30.41	0.14
35	*Astreopora myriophthalma*	17	0.15	369.40	21.72	80.50	0.35
36	*Montipora danae*	11	0.10	256.26	23.29	83.63	0.24
37	*Montipora effusa*	2	0.11	279.06	139.53	142.21	0.26
38	*Montipora eilatensis*	3	0.04	99.73	33.24	47.85	0.09
39	*Montipora meandrina*	62	1.07	2686.93	43.33	275.93	2.53
40	*Montipora monasteriata*	23	0.35	873.43	37.97	92.57	0.82
41	*Montipora stilosa*	61	2.10	5257.60	86.19	322.00	4.95
42	*Montipora venosa*	4	0.10	245.52	61.38	68.42	0.23
43	*Montipora sp.*	84	0.94	2425.75	28.87	181.57	2.28
44	*Gardineroseris ponderosa*	4	0.11	281.75	70.43	123.43	0.27
45	*Leptoseris sp.*	8	0.10	237.92	29.74	68.42	0.22
46	*Pachyseris speciosa*	1	0.01	31.75	31.75	31.75	0.03
47	*Pavona danae*	5	0.06	148.03	29.60	63.95	0.14
48	*Pavona decussata*	4	0.04	110.46	27.61	49.64	0.10
49	*Pavona varians*	41	0.64	1605.54	39.15	231.21	1.51
50	*Coscinarea monile*	4	0.14	347.49	86.87	168.60	0.33
51	*Cycloseris doederleini*	2	0.02	61.71	30.85	38.90	0.06
52	*Cycloseris sp.*	1	0.01	23.25	23.25	23.25	0.02
53	*Fungia fungites*	7	0.08	188.72	26.96	55.00	0.18
54	*Fungia granulosa*	6	0.05	130.59	26.11	40.25	0.12
55	*Alveopora daedalea*	24	0.35	872.09	36.33	139.08	0.82
56	*Goniopora planulata*	34	2.03	5080.05	149.41	1301.43	4.78
57	*Goniopora sp.*	12	0.24	587.66	48.97	177.54	0.55
58	*Porites (Synarea) convexa*	4	0.11	281.75	70.43	111.80	0.27
59	*Porites lutea*	11	0.23	571.10	51.91	152.95	0.54
60	*Porites sp.*	23	0.15	385.95	16.78	52.32	0.36
61	*Caulastrea tumida*	2	0.31	770.57	385.29	354.65	0.73
62	*Cyphastrea chalcidicum*	16	0.17	429.33	26.83	96.60	0.40
63	*Cyphastrea microphthalma*	27	0.28	690.51	25.57	55.00	0.65
64	*Cyphastrea serailia*	2	0.02	49.19	24.59	35.77	0.05
65	*Cyphastrea sp.*	1	0.01	17.88	17.88	17.88	0.02
66	*Echinopora gemmacea*	24	0.32	812.16	33.84	138.64	0.76
67	*Echinopora gemmacea fruticulosa*	8	0.11	268.33	33.54	137.29	0.25
68	*Echinopora lamellosa*	1	0.02	50.08	50.08	50.08	0.05
69	*Favia amicorum*	5	0.07	162.79	32.55	42.93	0.15
70	*Favia favus*	25	0.24	592.57	23.70	63.95	0.56

Appendix II - Table 1. continued.

Nr.	Order, Genus, Species	Number of colonies	Contribution to total 5x5m area (%)	Contribution to total 5x5m area (cm^2)	Average size of individual colony (cm^2)	Size of respective largest colony (cm^2)	Contribution to total live cnidarian cover (%)
71	*Favia laxa*	7	0.07	330.50	47.21	45.16	0.31
72	*Favia pallida*	3	0.03	77.81	25.93	40.25	0.07
73	*Favia stelligera*	9	0.20	505.36	56.15	125.67	0.48
74	*Favia sp.*	16	0.09	234.79	14.67	49.19	0.22
75	*Favites abdita*	7	0.09	232.55	33.22	49.19	0.22
76	*Favites peresi*	65	0.50	1239.26	19.06	64.40	1.17
77	*Favites sp.*	25	0.17	436.94	17.47	65.74	0.41
78	*Goniastrea edwardsi*	1	0.01	12.52	12.52	12.52	0.01
79	*Goniastrea pectinata*	162	1.68	4195.43	25.89	308.13	3.95
80	*Goniastrea retiformis*	27	0.35	880.58	32.61	111.80	0.83
81	*Goniastrea sp.*	7	0.13	328.26	46.89	90.78	0.31
82	*Hydnophora exaesa*	4	0.05	112.70	28.17	51.87	0.11
83	*Leptastrea bottae*	58	0.63	1573.34	393.33	178.89	1.48
84	*Leptastrea purpurea*	3	0.01	33.09	11.03	13.86	0.03
85	*Leptastrea transversa*	11	0.10	242.39	22.03	46.51	0.23
86	*Montastrea forskaelana*	15	0.26	639.53	42.63	206.61	0.60
87	*Platygyra lamellina*	14	0.19	487.47	34.81	95.25	0.46
88	*Galaxea fascicularis*	6	0.06	160.10	26.68	37.11	0.15
89	*Merulina ampliata*	1	0.01	24.15	24.15	24.15	0.02
90	*Acanthastrea echinata*	5	0.02	40.69	8.13	12.52	0.08
91	*Lobophyllia corymbosa*	2	0.03	63.50	31.75	33.09	0.06
92	*Lobophyllia costata*	8	0.16	402.50	50.31	129.69	0.38
93	*Lobophyllia hemprichi*	2	0.01	10.28	5.14	4.91	0.01
94	*Echinophyllia aspera*	1	0.12	292.03	292.03	292.03	0.27
95	*Mycedium tubifex*	18	0.26	638.19	35.45	69.76	0.60
96	*Turbinaria mesenterina*	4	0.05	121.64	30.41	78.26	0.11
	Zoantharia	3	0.03	65.29			0.06
97	*Palythoa tuberculosa*	3	0.03	65.29	21.76	42.48	0.06
	Crustose coralline algae	25	0.20	494.18	19.76	87.20	
	Sponges	7	0.17	429.33	61.33	211.98	
	Coral rock and rubble		30.27	76840.75			
	Sand		26.15	65383.24			



Appendix II - Figure 1. Coral community species composition as recorded and colour coded by Mergner and Schuhmacher (1981).

Appendix III - Table 1. Spearman rank correlation tests between status and process parameters for both seasons combined. Values are Spearman rank correlation coefficients; coefficients in italics are not significant. * = p<0.05, ** = p<0.01.

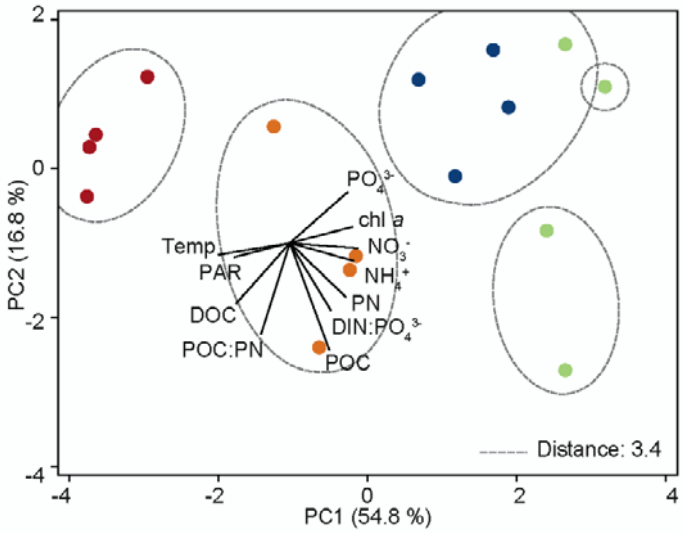
Temp	X												
Light	0.48*	X											
Chl a	-0.75**	-0.67**	X										
DOC	0.73**	*0.20*	-0.55**	X									
POC	*-0.03*	*0.13*	*0.07*	*-0.14*	X								
PN	-0.42*	*-0.03*	*0.35*	*-0.34*	0.66**	X							
NH4	-0.71**	*-0.09*	*0.40*	-0.72**	*-0.06*	*0.10*	X						
PO4	-0.73**	*-0.03*	0.48*	-0.56**	*-0.06*	*0.30*	0.68**	X					
NOx	-0.80**	-0.63**	0.63**	-0.57**	*-0.26*	*0.18*	0.60**	0.69**	X				
Pnet	-0.59**	-0.56**	0.60**	-0.41*	*-0.36*	*0.15*	*0.34*	0.47*	0.85**	X			
BOD	*0.32*	0.59**	-0.48*	*0.06*	*0.36*	*-0.11*	*0.02*	*-0.19*	-0.62**	-0.60**	X		
Pgross	-0.44*	*-0.29*	*0.34*	-0.44*	*-0.17*	*-0.05*	0.51**	*0.40*	0.63**	0.72**	*0.06*	X	
Nfix	*0.11*	0.59*	*-0.41*	*0.37*	-0.57*	*-0.31*	*-0.21*	*0.22*	*-0.02*	*0.19*	*0.17*	*0.23*	X
	Temp	Light	Chl a	DOC	POC	PN	NH4	PO4	NOx	Pnet	BOD	Pgross	Nfix

Appendix III - Table 2. Spearman rank correlation tests between status and process parameters for the mixed season. Values are Spearman rank correlation coefficients; coefficients in italics are not significant. * = p<0.05, ** = p<0.01.

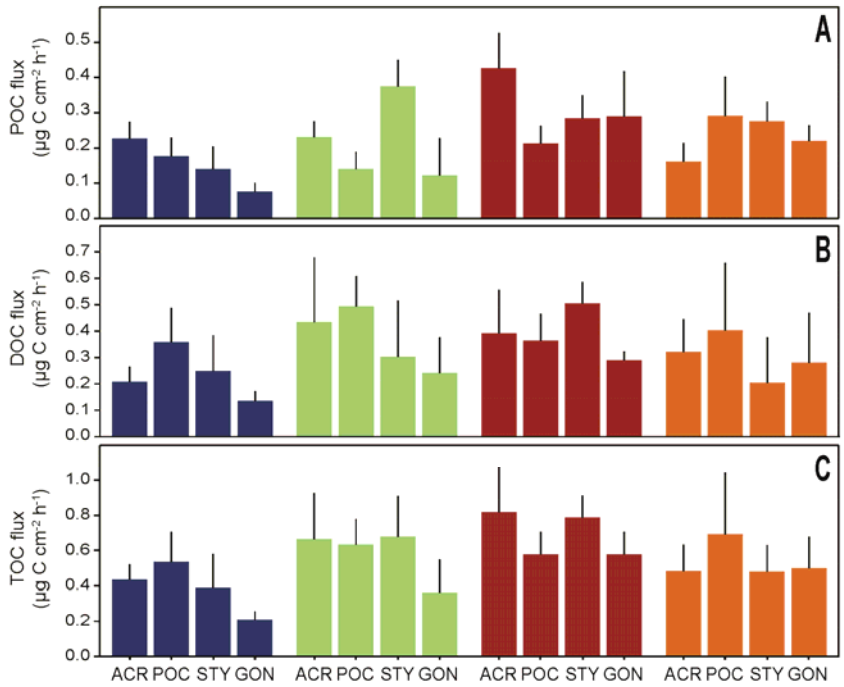
Temp	X												
Light	*0.15*	X											
Chl a	*0.02*	*-0.30*	X										
DOC	*-0.10*	*-0.01*	*-0.04*	X									
POC	*0.18*	*0.60*	*-0.14*	*-0.16*	X								
PN	*-0.07*	*0.34*	*-0.18*	*0.21*	0.76**	X							
NH4	*-0.21*	0.67*	*-0.26*	*-0.26*	*-0.01*	*-0.31*	X						
PO4	*-0.16*	*0.13*	*0.28*	*0.10*	*-0.22*	*-0.45*	*0.49*	X					
NOx	*-0.48*	-0.63*	*0.36*	*0.26*	-0.61*	*-0.53*	*-0.05*	*0.43*	X				
Pnet	*-0.15*	-0.79**	*0.38*	*0.06*	*-0.66**	*-0.56*	*-0.30*	*-0.04*	0.72**	X			
BOD	*0.15*	0.90**	*-0.35*	*-0.39*	*0.64**	*0.34*	*0.52*	*-0.18*	-0.71**	-0.63*	X		
Pgross	*-0.16*	*-0.07*	*0.05*	*-0.41*	*-0.27*	*-0.39*	*0.20*	*-0.22*	*0.18*	*0.55*	*0.20*	X	
Nfix	-0.79*	*<0.001*	*0.37*	*-0.14*	*-0.50*	*-0.54*	*0.61*	*0.36*	*0.54*	*0.39*	*0.18*	0.79*	
	Temp	Light	Chl a	DOC	POC	PN	NH4	PO4	NOx	Pnet	BOD	Pgross	Nfix

Appendix III - Table 3. Spearman rank correlation tests between status and process parameters for the stratified season. Values are Spearman rank correlation coefficients; coefficients in italics are not significant. * = p<0.05, ** = p<0.01.

Temp	X												
Light	0.92**	X											
Chl a	-0.86**	-0.90**	X										
DOC	*0.43*	*0.35*	*-0.32*	X									
POC	*-0.26*	*-0.29*	*0.29*	*-0.18*	X								
PN	*-0.08*	*-0.07*	*0.21*	*-0.06*	0.80**	X							
NH4	*-0.35*	*-0.41*	*0.12*	*-0.39*	*-0.14*	*-0.34*	X						
PO4	*0.24*	*0.20*	*-0.30*	*0.42*	*0.01*	*-0.27*	*-0.07*	X					
NOx	*-0.58*	*-0.60*	0.66*	*-0.17*	*0.27*	*0.13*	*0.16*	*-0.38*	X				
Pnet	*-0.32*	*-0.43*	*0.52*	*0.12*	*-0.06*	*0.18*	*0.15*	*-0.16*	0.67*	X			
BOD	*0.30*	*0.26*	*-0.50*	*-0.03*	*-0.10*	*-0.37*	*0.20*	0.58*	*-0.44*	*-0.32*	X		
Pgross	*-0.43*	*-0.55*	*0.41*	*-0.23*	*0.12*	*-0.02*	*0.51*	*0.19*	*0.52*	0.67*	*0.35*	X	
Nfix	0.89**	0.79*	*-0.75*	0.86*	*-0.75*	*-0.36*	*-0.71*	*0.57*	*-0.50*	*-0.11*	*0.46*	*-0.14*	X
	Temp	Light	Chl a	DOC	POC	PN	NH4	PO4	NOx	Pnet	BOD	Pgross	Nfix



Appendix IV - Figure 1. 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-}).



Appendix IV - Figure 2. 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 Appendix Table 3 and 6. Colours represent winter (blue), spring (green), summer (red) and autumn (orange).

Appendix IV - Table 1. 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)

Appendix IV - Table 2. 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				

Appendix IV - Table 3. 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	***

Appendix IV - Table 4. Results of *a posteriori* tests for the interaction term Season x Genus, if significant (cf. Appendix Table 3), 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.

Appendix IV - Table 5. Results of *a posteriori* tests for the interaction term Season x Genus, if significant (cf. Appendix Table 3), 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.	

Appendix IV - Table 6. Results of *a posteriori* tests for the factor Season, if the interaction term was not significant (cf. Appendix Table 3). 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.

Appendix IV - Supplementary methods: 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. Resulting fluxes are presented in Fig. 4.4 and Appendix Table 7.

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 (Muller-Parker *et al.* 1994), and a fraction of 0.1 of primary production fuelled by new N uptake (Rahav *et al.* 1989). 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.

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 (Muscatine *et al.* 1981). 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 (Muscatine *et al.* 1981). The per cent contribution of zooxanthellae-acquired C to daily animal respiration (CZAR) was calculated according to Muscatine *et al.* (1981), using T=0.78 according to Tremblay *et al.* (2012).

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 (Kayanne *et al.* 2005; Lesser *et al.* 2007), 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.* (2011) for an association between a N_2 fixer and a eukaryotic unicellular alga.

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) (Grottoli *et al.* 2006; Palardy *et al.* 2008; Tremblay *et al.* 2011) 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. Appendix Table 1). The

assumption of constant seasonal feeding was derived from non-variable seasonal observations of micro- and mesozooplankton depletion in local coral reef-overlying waters (Yahel *et al.* 2005), and from the locally constant, yet relatively insignificant, nanoplankton biomass (Lindell and Post 1995; Al-Najjar *et al.* 2007), which is accepted among pico- and nanoparticles as the most important contribution to C (84-94%) and N (52-85%) ingested by corals (Houlbrèque *et al.* 2004). 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. Appendix Table 1) and an average N assimilation efficiency (Bythell 1988; Anthony 1999; Mills 2000; Piniak *et al.* 2003; Mills *et al.* 2004) of 90%. A fraction of 0.2 of the calculated flux was assumed to be subsequently transferred from the host to the zooxanthellae (Cook 1972; Szmant-Froelich 1981; Piniak *et al.* 2003), 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.

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.* (2008), 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. Appendix Table 1) to DON concentrations applying DOC:DON=14 (Tanaka *et al.* 2011) and assuming DFAA=0.1*DON (Bronk 2002). A fraction of 0.33 of the calculated flux was assumed to be taken up by the zooxanthellae rather than by the host Grover *et al.* (2008), and thus regarded relevant for zooxanthellae N demand and CZND calculations.

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.* (2006), 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 (Bronk 2002). A fraction of 0.2 of the calculated flux was finally assumed to be uptaken by the zooxanthellae rather than by the host (Grover *et al.* 2006), and thus regarded relevant for zooxanthellae N demand and CZND calculations.

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.

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 and D'Elia (1978), 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. Appendix Table 1) and K is NH_4^+ concentration at half-maximal uptake rate (Muscatine and Porter 1977) (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. Fig. 4.2). 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 (Grover *et al.* 2002; Pernice *et al.* 2012) and CZND calculations.

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 (1990), where V is NO_3^- uptake rate (nmol N cm^{-2} h^{-1}) and $[NO_3]$ is *in situ* concentration (nmol l^{-1}) (cf. Appendix Table 1). As NO_3^- assimilation is exclusive to zooxanthellae (Crossland and Barnes 1977; Leggat *et al.* 2007; Kopp *et al.* 2013), the calculated flux was assumed to be taken up at a ratio of 1 for CZND calculations.

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 (Appendix Fig. 2) 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.* (2010) at the sampling site, and an average DOC:DON ratio of 18.6 obtained from literature (Tanaka *et al.* 2008; Tanaka *et al.* 2009; Tanaka *et al.* 2010). The per cent loss by TON release of the total acquired N (LON) was calculated by the equation: LON[%]=TONrelease/Σ(N uptake rates)*100.

Appendix IV - Table 7. Parameters used to build the C and N flux model.

	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 respiration (CZAR, %)	110 ± 18	86 ± 14
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

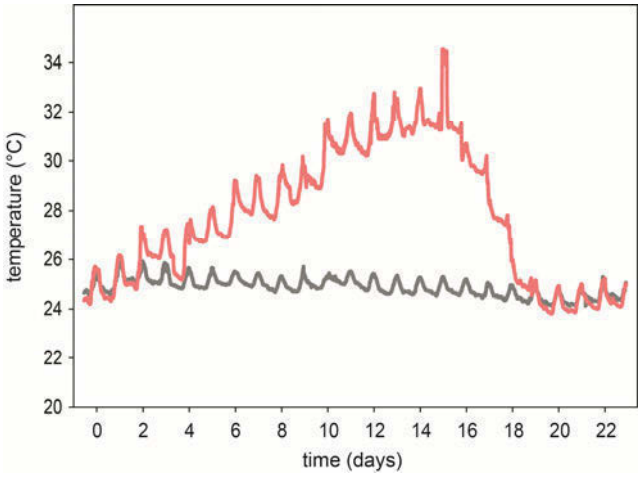
Parameters are either obtained from data sets generated by the present study (*) or calculated as described in the Supplementary Methods section. Values are reported as means ± standard deviation.

Appendix V - Table 1. 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.

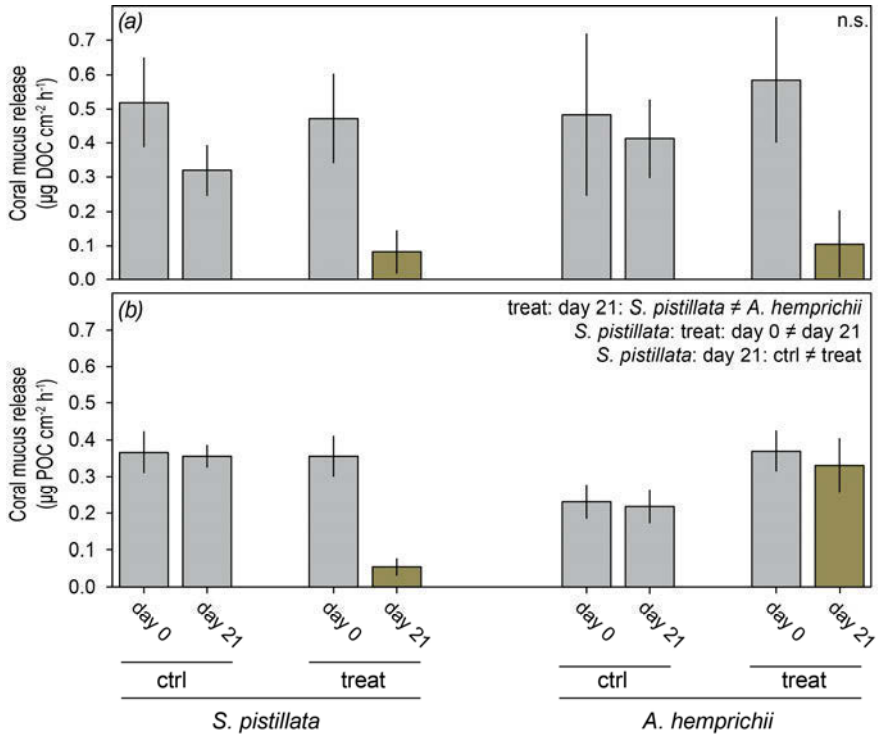
Parameter	Factor	*df*, $df_{residual}$	F	*p*
N_2 fixation	Season	6, 83	5.78	<0.001
	Substrate	3, 83	60.09	<0.001
	Season * Substrate	2, 77	299.37	<0.001
P_{gross}	Season	3, 79	12.68	<0.001
	Substrate	2, 79	63.64	<0.001
	Season * Substrate	5, 74	3.96	<0.001
R	Season	3, 89	18.00	<0.001
	Substrate	2, 89	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

Appendix V - Table 2. 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



Appendix VI - Figure 1. Temperature profiles for the two thermal treatments with which *Stylophora pistillata* and *Acropora hemprichii* nubbins were exposed. 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.



Appendix VI - Figure 2. POC and DOC fluxes of the coral nubbins at different times during the experiment. Values are means ± SEM 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; dark green bars, ambient temperature after exposure to 34°C. Results of the PERMANOVAs are reported in Appendix Table 2 and 3.

Appendix VI - Figure 3. *Stylophora pistillata* after the exposure to 34 °C, with open polyps at night.

Appendix VI - Table 1. Dissolved inorganic nutrients and light intensity over the course of the manipulation experiment. Values are means ± SEM calculated from weekly sampling of inorganic nutrients (n = 4) and maximum daily averages (11:30 - 14:30) of light intensity (n = 22).

Environmental variable	Control	Treatment	*In situ*
Light intensity (PAR)	279 (74)	297 (57)	283 (19)
Ammonium (μM)	0.15 (0.03)	0.14 (0.02)	0.18 (0.04)
Phosphate (μM)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)
Nitrogen oxides (μM)	0.30 (0.16)	0.27 (0.18)	0.27 (0.13)

Appendix VI - Table 2. 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.

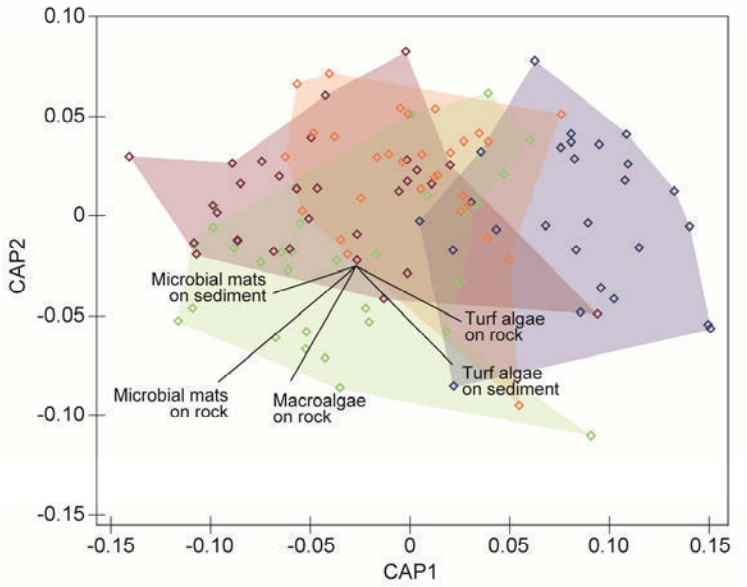
Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
P_N : R_D ratio	Species (Sp)	1	307	307	21.5	**0.0002**	9922
	Treatment (Tr)	1	611	611	42.7	**0.0001**	9928
	Time (Ti)	2	244	122	8.5	**0.0009**	9947
	SpxTr	1	294	294	20.6	**0.0001**	9932
	SpxTi	2	745	373	26.1	**0.0001**	9961
	TrxTi**	1	572	572	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				
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				

Appendix VI - Table 2. continued.

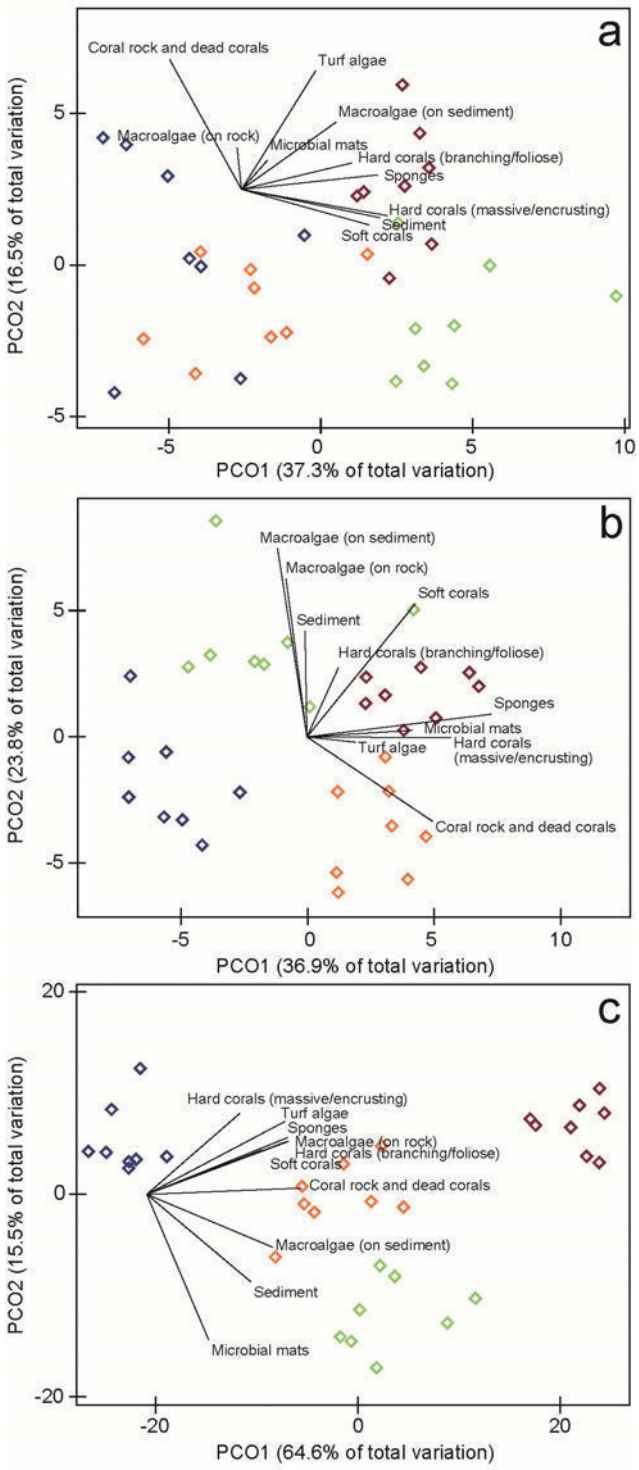
Variable	Effect	*df*	SS	MS	Pseudo-*F*	*p* value	Unique perms
Mucus Release (POC)	Species (Sp)	1	317	317	1.5	0.2065	9942
	Treatment (Tr)	1	404	404	2.0	0.1469	9928
	Time (Ti)	1	1742	1742	8.5	**0.0021**	9947
	SpxTr	1	2716	2716	13.2	**0.0003**	9927
	SpxTi	1	1198	1198	5.8	**0.0094**	9948
	TrxTi**	1	1369	1369	6.6	**0.0065**	9940
	SpxTrxTi**	1	1072	1072	5.2	**0.0127**	9935
	Residuals	52	10715	206			
	Total	59	20180				

Appendix VI - Table 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				
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				



Appendix VII - Figure 1. Canonical Analysis of Principal Coordinates (CAP) plot showing differences in benthic community composition between seasons. Vectors are overlaid for benthic components that have a Spearman rank correlation greater than 0.4 with either of the resulting CAP axes. Colours represent winter (blue), spring (green), summer (red) and autumn (orange).



Appendix VII - Figure 2. PCO plots showing differences in benthic community gross primary production (a) respiration (b) and N_2 fixation (c), between seasons. Benthic categories correlation vectors are overlaid on the plots to visualise relationships between variables and the ordination axes. Colours represent winter (blue), spring (green), summer (red) and autumn (orange).

Appendix VII - Table 1. Cover (%) of all benthic categories at each reef habitat in all seasons. Values are means (n = 6) with SE in parentheses.

Benthic category	Season	Habitat				
		Forereef	Transition zone	Sand belt	Reef crest	Reef flat
Hard corals (branching/ foliose)	Winter	15.3 (1.7)	15.7 (2.0)	10.5 (1.9)	15.5 (2.4)	5.8 (3.0)
	Spring	17.3 (3.4)	14.8 (3.1)	6.8 (2.4)	13.3 (3.2)	6.0 (2.5)
	Summer	13.2 (3.9)	14.5 (2.9)	7.8 (1.7)	13.8 (3.0)	7.5 (3.1)
	Autumn	18.1 (1.9)	16.0 (1.9)	7.8 (2.0)	13.7 (1.8)	7.2 (1.7)
Hard corals (massive/ encrusting)	Winter	45.3 (3.4)	15.8 (7.2)	10.7 (6.8)	17.2 (2.1)	14.2 (1.8)
	Spring	37.0 (1.8)	20.5 (2.6)	8.3 (2.5)	13.5 (2.8)	13.2 (3.3)
	Summer	44.3 (4.5)	23.7 (4.6)	7.5 (4.1)	20.0 (8.6)	16.8 (8.4)
	Autumn	39.3 (3.8)	21.3 (3.3)	6.5 (4.0)	19.3 (2.6)	20.7 (2.1)
Soft corals	Winter	10.0 (1.5)	13.3 (2.0)	1.8 (3.0)	3.8 (3.9)	5.0 (4.6)
	Spring	7.0 (3.8)	14.6 (0.7)	3.2 (1.0)	5.5 (1.0)	5.3 (1.1)
	Summer	8.2 (1.0)	10.5 (1.2)	3.3 (1.1)	3.0 (0.8)	10.3 (2.2)
	Autumn	11.2 (1.6)	14.8 (2.6)	2.7 (4.1)	7.3 (4.2)	10.8 (4.3)
Coral rock ('bare')	Winter	8.2 (2.3)	8.8 (2.6)	9.7 (2.5)	22.1 (1.9)	17.5 (1.7)
	Spring	7.3 (2.0)	7.2 (2.5)	7.3 (4.1)	19.3 (4.4)	14.3 (2.8)
	Summer	8.5 (3.6)	7.8 (2.7)	7.7 (2.1)	21.3 (2.6)	20.7 (1.3)
	Autumn	9.2 (1.8)	5.2 (1.9)	8.0 (1.8)	20.7 (0.7)	15.7 (1.3)
Dead corals ('bare')	Winter	1.5 (0.9)	1.7 (1.1)	2.0 (0.8)	0.8 (0.7)	6.9 (0.6)
	Spring	1.8 (0.7)	1.3 (0.9)	1.0 (0.6)	0.2 (0.3)	2.3 (0.3)
	Summer	1.2 (0.4)	1.8 (2.0)	0.8 (2.3)	2.3 (2.0)	3.3 (0.6)
	Autumn	1.8 (0.5)	1.8 (0.5)	1.5 (0.4)	2.3 (0.4)	1.5 (0.5)
Microbial mats (on rock)	Winter	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.5 (0.0)
	Spring	4.5 (0.0)	1.3 (0.0)	3.3 (0.0)	5.8 (0.0)	2.0 (0.0)
	Summer	2.5 (0.6)	3.0 (1.0)	1.0 (1.1)	0.8 (1.2)	6.7 (1.4)
	Autumn	2.5 (1.3)	0.5 (1.4)	0.3 (1.0)	0.0 (0.6)	0.7 (0.8)
Turf algae (on rock)	Winter	6.5 (1.8)	8.0 (1.6)	3.7 (1.2)	12.0 (1.9)	10.8 (1.8)
	Spring	3.3 (2.0)	3.6 (1.1)	2.2 (2.2)	8.8 (2.6)	15.5 (1.2)
	Summer	3.0 (2.5)	2.2 (3.5)	1.5 (6.2)	5.7 (5.6)	3.8 (5.4)
	Autumn	8.0 (1.4)	2.7 (1.3)	1.7 (1.0)	3.0 (1.0)	12.7 (1.0)
Macroalgae (on rock)	Winter	2.7 (1.3)	4.2 (1.2)	3.5 (1.6)	6.7 (1.7)	4.0 (2.2)
	Spring	10.8 (2.0)	6.9 (1.8)	7.2 (2.3)	13.8 (2.8)	6.0 (3.0)
	Summer	7.8 (2.9)	5.0 (2.3)	3.7 (2.2)	7.7 (3.0)	1.8 (2.6)
	Autumn	1.7 (2.2)	3.2 (1.9)	3.0 (1.8)	9.5 (1.3)	3.0 (2.0)
Sponges	Winter	0.5 (0.3)	0.7 (0.3)	0.7 (0.3)	0.0 (0.3)	0.0 (0.2)
	Spring	0.5 (0.2)	0.5 (0.2)	0.7 (0.2)	0.0 (0.2)	0.0 (0.0)
	Summer	1.0 (0.0)	0.7 (0.0)	1.0 (0.0)	0.0 (0.2)	0.0 (0.2)
	Autumn	1.2 (0.3)	1.0 (0.3)	1.2 (0.3)	0.3 (0.3)	0.2 (0.4)
Sediment ('bare')	Winter	6.2 (3.2)	23.8 (5.7)	37.7 (6.0)	1.7 (3.6)	15.7 (3.6)
	Spring	7.5 (4.9)	25.0 (4.9)	50.3 (8.8)	0.7 (9.3)	16.8 (0.9)
	Summer	6.2 (3.2)	26.3 (3.7)	56.2 (1.0)	0.7 (3.5)	7.3 (4.5)
	Autumn	4.7 (3.5)	29.5 (4.9)	57.8 (5.4)	0.2 (4.2)	9.5 (6.8)
Microbial mats (on sediment)	Winter	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.3 (0.0)
	Spring	0.3 (0.0)	0.3 (0.0)	1.3 (0.0)	0.0 (0.0)	0.2 (0.0)
	Summer	0.8 (0.1)	0.8 (0.2)	1.7 (0.2)	0.0 (0.2)	0.0 (0.2)
	Autumn	0.3 (0.2)	0.5 (0.2)	1.7 (0.1)	0.0 (0.2)	0.0 (0.5)

Appendix VII - Table 1. continued.

Benthic category	Season	Habitat				
		Forereef	Transition zone	Sand belt	Reef crest	Reef flat
Turf algae (on sediment)	Winter	0.7 (0.5)	4.3 (2.1)	12.8 (2.2)	0.0 (2.2)	6.1 (1.3)
	Spring	0.2 (3.4)	0.0 (5.7)	0.5 (5.9)	0.0 (6.0)	1.0 (0.0)
	Summer	0.0 (0.6)	0.2 (1.7)	0.0 (1.7)	0.0 (1.8)	0.0 (1.0)
	Autumn	0.3 (0.2)	0.3 (0.2)	0.7 (0.0)	0.0 (0.0)	0.0 (0.0)
Macroalgae (on sediment)	Winter	0.0 (0.0)	0.3 (0.0)	0.3 (0.3)	0.0 (0.3)	0.3 (0.3)
	Spring	0.0 (0.0)	1.3 (0.3)	0.7 (0.3)	0.0 (0.3)	0.2 (0.0)
	Summer	0.5 (0.1)	0.8 (0.1)	0.2 (0.2)	0.2 (0.2)	0.0 (0.2)
	Autumn	0.2 (0.0)	0.3 (0.1)	0.2 (0.2)	0.0 (0.8)	0.7 (0.8)
Millepora	Winter	0.0 (0.0)	0.0 (0.0)	0.3 (0.0)	13.3 (0.0)	0.0 (0.0)
	Spring	0.0 (0.0)	0.0 (0.3)	0.3 (2.8)	9.0 (2.4)	0.2 (1.0)
	Summer	0.0 (2.9)	0.0 (3.8)	0.7 (0.0)	11.0 (0.0)	0.2 (0.0)
	Autumn	0.0 (0.0)	0.2 (0.0)	0.8 (0.0)	8.5 (0.0)	0.5 (0.0)
Crustose coralline algae (CCA)	Winter	1.2 (0.2)	0.2 (0.3)	1.7 (0.3)	3.5 (0.2)	3.3 (1.0)
	Spring	0.0 (1.0)	0.0 (0.9)	0.0 (1.2)	5.2 (1.6)	6.0 (1.6)
	Summer	0.0 (1.6)	0.0 (1.6)	0.0 (1.6)	7.8 (1.6)	10.8 (1.2)
	Autumn	0.2 (0.0)	0.0 (0.0)	0.0 (0.0)	7.7 (0.0)	9.0 (0.0)
Zoanthids	Winter	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.0 (0.0)	1.0 (0.0)
	Spring	0.2 (0.0)	0.2 (0.0)	0.0 (0.3)	2.0 (0.4)	3.7 (0.4)
	Summer	0.0 (0.5)	0.0 (0.6)	0.2 (0.6)	2.5 (0.6)	4.5 (0.2)
	Autumn	0.2 (0.2)	0.2 (0.2)	0.3 (0.2)	2.8 (0.2)	4.5 (0.0)
Coral rubble	Winter	1.5 (0.5)	3.0 (0.8)	4.5 (0.8)	0.7 (0.7)	6.1 (0.7)
	Spring	2.0 (0.6)	2.6 (0.8)	6.5 (1.2)	2.3 (1.3)	4.8 (0.7)
	Summer	2.8 (1.4)	2.3 (1.4)	6.7 (0.6)	3.0 (1.4)	4.3 (1.0)
	Autumn	1.3 (0.5)	2.5 (0.4)	5.8 (0.3)	3.2 (0.4)	1.3 (0.8)
Others	Winter	0.5 (0.3)	0.2 (0.2)	0.2 (0.2)	1.8 (0.2)	1.8 (0.0)
	Spring	0.2 (0.2)	0.3 (0.2)	0.3 (0.5)	0.5 (0.6)	2.5 (0.4)
	Summer	0.0 (0.4)	0.3 (0.3)	0.2 (0.4)	0.2 (0.5)	1.8 (0.6)
	Autumn	0.0 (0.2)	0.0 (0.1)	0.0 (0.2)	1.5 (0.2)	2.2 (0.2)

Appendix VII - Table 2. Zonal statistics of each habitat, and percentage of area covered by the benthic categories investigated. Values are means with SD in parentheses.

Habitat	Mean depth ± SD (m)	Max depth (m)	Min depth (m)	Water volume (m^3)	Planar 2D area (m^2)	Slope 2D area (m^2)	2D area covered by benthic categories investigated (%)
Reef flat	0.7 (0.2)	2.2	0.5	38318	54214	54244	82.9 (3.9)
Reef crest	2.2 (1.0)	5.9	0.5	34541	15831	16071	78.1 (2.6)
Sand belt	5.2 (2.2)	15.6	1.0	135294	26186	26705	92.9 (0.4)
Transition zone	9.0 (2.9)	17.4	3.2	422929	46825	47997	97.0 (0.3)
Forereef	16.7 (2.6)	20.6	7.0	592619	35395	36616	97.5 (0.7)
Total reef area	6.8 (3.7)	20.6	0.5	1223700	178451	181633	89.7 (8.2)

Appendix VII - Table 3. Description of the incubated categories, replication for each season and calculated 2D:3D conversion factors (where applicable).

Benthic category	Replication	2D:3D	Description / taxa incubated
Hard corals (branching/foliose)	$n = 24$	7.6	Acropora, Stylophora, Pocillopora
Hard corals (massive/encrusting)	$n = 8$	2.9	Goniastrea
Soft corals	$n = 16$	6.5	Xenidae, Sarcophyton
Coral rock ('bare')	$n = 8$	2.5	Biogenic reef framework with the carbonate structure clearly visible
Dead corals ('bare')	-	5.2	Assumed equal to 'bare' coral rock
Microbial mats (on rock)	-	3.9	Assumed equal to microbial mats on sediment
Turf algae (on rock)	$n = 8$	3.9	Dense mats composed of a heterogeneous assemblage of filamentous algae
Macroalgae (on rock)	$n = 8$	3.9	Lobophora
Sponges	$n = 8$	3.9	Mycale
Sediment ('bare')	$n = 8$	1.0	Top 1 cm surface layer with its microphytobenthos
Microbial mats (on sediment)	$n = 8$	1.0	Top 1 cm surface layer covered by the mat
Turf algae (on sediment)	-	1.0	Assumed equal to turf algae on rock
Macroalgae (on sediment)	$n = 8$	1.8	Caulerpa
Seawater	$n = 32$	-	800 mL water volume per replicate

Appendix VII - Table 4. Gross primary production (GPP), respiration (R) and N_2 fixation (DNF) of the benthic categories investigated in each season, after considering their respective three-dimensional structure (2D:3D), in mmol (C or N) m^{-2} benthos 3D area d^{-1}. Values are means (*n* = see Appendix Table 3) with SE in parentheses.

Benthic category	Season	GPP	R	DNF
Hard corals (branching/foliose)	Winter	648 (62)	424 (38)	0.035 (0.010)
	Spring	754 (26)	403 (19)	0.076 (0.014)
	Summer	733 (31)	500 (22)	0.303 (0.046)
	Autumn	618 (38)	393 (33)	0.050 (0.011)
Hard corals (massive/encrusting)	Winter	239 (16)	147 (9)	0.028 (0.010)
	Spring	342 (7)	153 (11)	0.017 (0.006)
	Summer	326 (17)	225 (17)	0.194 (0.040)
	Autumn	299 (23)	182 (7)	0.099 (0.033)
Soft corals	Winter	256 (23)	138 (8)	0.013 (0.004)
	Spring	403 (14)	208 (8)	0.021 (0.008)
	Summer	344 (15)	231 (14)	0.078 (0.012)
	Autumn	300 (21)	162 (9)	0.012 (0.003)
Coral rock ('bare')	Winter	145 (25)	58 (12)	0.136 (0.046)
	Spring	85 (22)	42 (8)	0.942 (0.121)
	Summer	168 (16)	109 (20)	2.113 (0.289)
	Autumn	136 (14)	88 (8)	0.804 (0.118)
Dead corals ('bare')	Winter	298 (53)	121 (24)	0.280 (0.094)
	Spring	175 (45)	87 (17)	1.944 (0.251)
	Summer	347 (34)	225 (42)	4.362 (0.596)
	Autumn	280 (30)	181 (15)	1.660 (0.243)
Microbial mats (on rock)	Winter	365 (66)	100 (11)	2.450 (0.439)
	Spring	400 (33)	121 (11)	9.535 (0.522)
	Summer	386 (40)	142 (12)	4.140 (0.328)
	Autumn	355 (32)	134 (13)	5.335 (0.410)
Turf algae (on rock)	Winter	246 (14)	88 (4)	0.828 (0.078)
	Spring	256 (18)	73 (6)	1.234 (0.206)
	Summer	417 (27)	111 (9)	5.207 (0.449)
	Autumn	237 (14)	88 (6)	1.263 (0.088)
Macroalgae (on rock)	Winter	192 (14)	55 (3)	0.082 (0.045)
	Spring	179 (24)	86 (18)	0.268 (0.110)
	Summer	184 (12)	68 (4)	1.045 (0.189)
	Autumn	130 (10)	42 (6)	0.268 (0.061)
Sponges	Winter	30 (8)	221 (13)	0.011 (0.001)
	Spring	139 (37)	407 (89)	0.037 (0.013)
	Summer	143 (18)	524 (18)	0.260 (0.042)
	Autumn	80 (18)	526 (35)	0.058 (0.014)
Sediment ('bare')	Winter	17 (3)	15 (2)	0.142 (0.030)
	Spring	66 (10)	22 (4)	0.630 (0.067)
	Summer	37 (4)	18 (1)	0.518 (0.079)
	Autumn	20 (2)	11 (1)	0.137 (0.022)

Appendix VII - Table 4. continued.

Benthic category	Season	GPP	R	DNF
Microbial mats (on sediment)	Winter	94 (17)	26 (3)	0.631 (0.113)
	Spring	103 (8)	31 (3)	2.455 (0.134)
	Summer	99 (10)	37 (3)	1.066 (0.084)
	Autumn	91 (8)	34 (3)	1.374 (0.105)
Turf algae (on sediment)	Winter	63 (4)	23 (1)	0.213 (0.020)
	Spring	66 (5)	19 (2)	0.318 (0.053)
	Summer	107 (7)	29 (2)	1.341 (0.116)
	Autumn	61 (4)	23 (2)	0.325 (0.023)
Macroalgae (on sediment)	Winter	233 (31)	36 (5)	0.003 (0.001)
	Spring	258 (37)	73 (8)	0.445 (0.161)
	Summer	229 (15)	49 (4)	0.358 (0.055)
	Autumn	169 (20)	19 (3)	0.157 (0.038)

Appendix VII - Table 5. Results of PERMANOVA looking at differences in benthic community cover among seasons and reef habitats. Shown are the main test (a) and the comparisons amongst seasons (b) and amongst reef habitats (c). Significant *p* values have a grey background.

	Source	*df*	SS	MS	Pseudo *F*	*P*(perm)
(a)	Season (Se)	3	5035.9	1678.6	5.8197	0.0001
	Reef habitat (Rh)	4	42437	10609	36.782	0.0001
	Se x Rh	12	4048	337.34	1.1695	0.183
	Residuals	98	28267	288.44		
	Total	117	79788			
(b)	Winter	Winter				
	Spring	0.0001	Spring			
	Summer	0.0001	0.0213	Summer		
	Autumn	0.0005	0.0002	0.0107	Autumn	
(c)	Reef flat	Reef flat				
	Reef crest	0.0001	Reef crest			
	Sand belt	0.0001	0.0001	Sand belt	Transition	
	Transition zone	0.0001	0.0001	0.0001	zone	
	Forereef	0.0001	0.0001	0.0001	0.0001	Forereef

Appendix VII - Table 6. Results of PERMANOVAs looking at differences in (a) gross primary production, (b) respiration and (c) N_2 fixation of the benthic community among the seasons, with the respective comparisons amongst seasons. Significant *p* values have a grey background.

	Source	*df*	SS	MS	Pseudo *F*	*P*(perm)
(a)	Season	3	621.56	207.19	6.9098	0.0001
	Residuals	28	839.57	29.984		
	Total	31	1461.1			
	Winter	Winter				
	Spring	0.0003	Spring			
	Summer	0.0001	0.0001	Summer		
	Autumn	0.0132	0.0003	0.0005	Autumn	
(b)	Season	3	833.49	277.83	10.649	0.0001
	Residuals	28	730.5	26.089		
	Total	31	1564			
	Winter	Winter				
	Spring	0.0003	Spring			
	Summer	0.0002	0.0002	Summer		
	Autumn	0.0005	0.0003	0.0006	Autumn	
(c)	Season	3	10224	3407.9	33.877	0.0001
	Residuals	28	2816.6	100.59		
	Total	31	13040			
	Winter	Winter				
	Spring	0.0004	Spring			
	Summer	0.0002	0.0001	Summer		
	Autumn	0.0003	0.0002	0.0003	Autumn	

Technical notes

Universität Bremen

Erklärung

Bremen, 14 Januar 2015

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

Dinitrogen fixation in coral reef ecosystems facing climate change

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

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

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Ulisse Cardini