Coral-associated microbial communities in reef-building corals of Ningaloo Reef Western Australia

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This thesis is submitted to Murdoch University
for the degree of Doctor of Philosophy

School of Biology & Biotechnology
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Submitted May 2011

To Coral Reefs

I declare this thesis to be a record of my own research, which has not been presented for the award of a degree at any other university.

Janja Ceh

Abstract

Coral reefs are at risk and human-induced environmental stressors in synergism with microorganisms have been shown to be the key players for their deterioration. Little is known about the dynamics of coral-microbial associations through different life stages of the coral holobiont and virtually nothing is known about coral-microbial partners in Western Australian coral reef systems. This project intended to investigate the presence, diversity, community structure and role of coral-associated microbes in Ningaloo Reef spawning and brooding corals. Different coral life stages were assessed.

To determine 'normal ranges' of coral-associated microbes, three coral species (*Acropora tenuis*, *Pocillopora damicornis* and *Favites abdita*) were tagged and examined over a period of one year, with sampling deployed every three months. One coral species was additionally sampled on Rottnest Island, 1200km south of Ningaloo Reef, to provide comparisons between coral-associated microbes in different geographical areas. The community structure of the coral-associated microorganisms was analysed by phylogenetic analysis of 16S rRNA gene clone libraries. Principal component analysis (PCA) revealed that samples grouped according to time and not species, indicating that coral-microbial associations may be a result of environmental drivers such as oceanographic characteristics, benthic community structure and temperature. Tissue samples from Rottnest Island corals revealed similarities in bacteria to the samples at Ningaloo Reef. This study highlights that coral-associated microbial communities are highly diverse; however, the complex interactions that determine the stability of these associations are not necessarily dependant on coral host specificity.

Reproduction plays a crucial role in the survival of species, therefore, data was acquired from three adult coral colonies, A*cropora tenuis* (broadcast spawner), *Pocillopora damicornis* (brooder) and *Tubastrea faulkneri* (ahermatypic), before and after coral mass spawning to determine if and through which drivers coral microbial communities changed through this event. A contemporary 454 sequencing approach was implemented and results revealed distinct bacterial shifts through coral mass spawning for all corals, independently of reproductive activity. Clear changes in bacterial assemblages were also detected for brooders after planulation. This infers that coral-associated microbial communities change through a coral mass spawning event and are likely driven by environmental factors and the respective bacterial community in the seawater, as well as by actual coral reproduction. Differences in coral-microbial communities reflected different life styles between brooding and spawning corals. Most *α-Proteobacteria* increased in abundance after spawning as well as after planulation, suggesting that specific bacteria are involved in coral reproduction irrespective of reproductive strategies; particularly bacteria affiliated with the *Roseobacter* clade followed this pattern.

The assessment of seawater collected from the broadcast spawning coral *A. tenuis* and *P. damicornis* after spawning and planulation, respectively revealed that adult corals, irrespective of their reproductive strategy release bacteria with their offspring which likely increases the fitness in the following processes involved in settlement and survival. Species affiliated with the genera *Roseobacter* and *Alteromonas* appear to play important roles in coral reproduction and early life history in corals.

Isolates from *P. damicornis* planulae were mainly affiliated with the genera *Vibrio* and *Alteromonas* and were found to be similar to bacteria released by the mother colony during planulation.

Finally the establishment of coral-microbial partnerships in coral larval stages and the potential role of these symbiotic relationships were studied. The early onset of bacterial associations

in brooding and broadcast spawning corals was visualized, exploring bacterial presence and their location in the coral organism, determining when and how bacteria enter coral tissues and their cycling of nutrients towards the coral-symbiotic algal partners. Nano-scale Second Ion Mass Spectrometry (SIMS) was applied to detect, image and map the uptake and translocation of ^{15}N from bacteria into coral larvae on a sub-cellular level. The study also combined Fluorescent *In Situ* Hybridisation (FISH) to co-localize the labelled substrate with bacteria and Transmission Electron Microscopy (TEM) to allow for ultra-structural resolution images to provide high resolution images. This study for the first time demonstrated the beneficial role of specific bacteria in translocating nitrogen into the coral holobiont, which is particularly important in the nutrient-poor environments corals live in.

Acknowledgements

I'm feeling privileged to have met and worked with some amazing people and it is my pleasure to express my gratitude to everyone who made this PhD-adventure possible and so enjoyable!

I'm incredibly grateful to my wonderful supervisors and friends Mike van Keulen and David Bourne. Their unconventional ways and 'nothing is impossible attitude' opened a lot of space for creativity and 'out of the box thinking'. Thanks for sharing your knowledge, resources and great times.

Thanks beyond words go to my friend Jean-Baptiste Raina for exposing me to his generosity, humor, honest opinions, statistics wizardry, excellent taste of music and for sharing my passion for science and coral reefs. I loved sharing a lab bench with you – even though you constantly 'displaced' my lab equipment.

I also want to thank people at Murdoch University for their patient assistance, Graham O'Hara, Gordon Thompson, and Michael Taylor. Special thanks go to Leila Eshraghi for sharing her lab space when I was in desperate need of some and for being such a nice person.

I'm happy to express my appreciation for all my volunteers in the field: Joetta Perrett, Sam Wells, Kim Mars, Swaantje Bennecke and Bernard O'Reilly. Thanks to Bruce Portman for letting me transform his beautiful yacht Pi Phrontis into a research vessel for a while. I specially want to thank Frazer McGregor, who not only provided his incredible knowledge and time to take me to suitable sampling sites, but also showed me amazing snorkeling spots and introduced me to some of his wondrous manta ray friends. Thanks Frazer – you're awesome!

I also want to thank my colleagues at the Australian Institute of Marine Science: Sebastian Schmidt and Andrew Negri & team for providing coral larvae, Rochelle Soo and Andy Miurhead for help in the lab, Beth Ballment for making me wear my lab coat, Nicole Koestner who volunteered counting thousands of coral larvae and Kim Lema and Eneour Puill-Stephan for introducing me to the wonderful world of kite surfing.

Thank you to everyone at the Centre of Microscopy and Characterization (University of Western Australia): Lyn Kirilak for her patience and sharing little microscopy secrets, John Murphy for helping with sections and keeping me entertained with interesting conversations about literature, cinematography and traveling, Peter Duncan for his constant good mood, Jeremy Shaw and Peta Clode for scientific advice, and the great NanoSIMS team: Matt Kilburn for his kindness, wit and intelligence and John Cliff for sharing his broad scientific knowledge and providing the needed cynicism to foresee reviewers' criticism. I truly enjoyed working with you.

I would also like to express my gratitude to people at the Wagner lab (Department of Microbial Ecology) for inviting me to conduct my FISH work in Vienna; special thanks to Markus Schmidt, Christoph Boehm and Faris Behnam for help in the lab and to Michi Wagner for fruitful advice and conversation.

The inspiration for this research was sparked by the work of Eugene Rosenberg, and Yossi Loya who exposed me to it. Thanks for that!

This research was made possible by the support of Murdoch University, the Australian Institute of Marine Science, the Western Australian Marine Science Institution and the Australian Microscopy & Microanalysis Research Facility.

Thanks for providing cozy work space, delicious food, great coffee and suitable entertainment through the last weeks of my thesis writing go to cafes in Fremantle: the X-Wray, the Wild Poppy and the Ootong and Lincoln; and thanks to my former housemate David Robertson for serenading through the chimney in the endless hours when I was blasting my sequences, and to an anonymous neighbour for so generously sharing their wireless internet.

Much appreciation goes to my parents Franc & Milka Ceh who planted the seeds of curiosity in me and to my brother Michael Ceh for all the laughter.

Finally I would like to thank Bernie O'Reilly – without you and all our little adventures life wouldn't be as much fun!

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CHAPTER 1

Introduction

1.1. Declining coral reefs

Coral reefs are amongst nature's most beautiful and wondrous ecosystems and have been forming the largest living structures on the planet for the past 200 million years. They have been built by individual coral animals of the phylum Cnidaria, well-adapted and adaptable organisms that have survived dramatic environmental changes in the past. Currently however, coral reefs have been degrading at an alarming rate over the past few decades (Baker et al. 2008; Hughes et al. 2003) and are facing risk of further and even more rapid decline in the near future (Carpenter et al. 2008) with prediction of approximately 60% of the world's corals being irretrievably lost by 2030.

The impact of anthropogenically-driven stressors on coral reef ecosystems is variable and occurs on both local and global scales. Major local human induced impacts on coral reefs include overfishing, nutrient enrichment, and the physical destruction of reefs. On a global scale, climate change imposes measurable effects and a major threat to coral reefs. Increases in atmospheric concentrations of carbon dioxide contribute to ocean warming as well as ocean acidification (Doney et al. 2009; Feely et al. 2009; Harvell et al. 2007), with both of these changes disrupting the balance of the complex multi-partner symbiotic relationships (De'ath et al. 2009; Kleypas and Yates 2009; Rohwer 2010) in coral reef ecosystem. Each of these stressors and their synergistic effects can result in coral stress or disease and ultimately in coral death (Hoegh-Guldberg et al. 2007).

1.2 The Coral Holobiont

Until recently, corals have been considered a product of a mutualistic interaction between corals and single cell algae symbionts belonging to the genus *Symbiodinium* (commonly referred to as zooxanthellae). This view has disregarded the symbiotic potential of the large, diverse and sometimes specific populations of other coral-associated micro-organisms including bacteria, viruses, fungi and Archaea, which all form a collaborative consortium named the coral holobiont (Knowlton and Rohwer 2003). While the zooxanthellae have been known as obligate partners supplying the majority of the coral host's metabolic requirements for carbon in photosynthate and contributing to the formation of the skeleton (Muscatine and Weis 1992), the roles of coral-associated microorganisms have not been well elucidated yet.

1.3 Coral-Microbial Partners

Micro-organisms are the most diverse and numerous organisms associated with reef building corals with over 100 million microbes estimated on a square centimetre of a healthy coral (Paul et al. 1986) and almost a billion microbes and 10 billion viruses per litre of sea water (Bowler et al. 2009). Bacterial assemblages are known to play an essential role in ecosystem functioning, driving biogeochemical cycles and ecological processes (Balser et al. 2007) and are likely to fulfil important functions within coral reef environments and more specifically in close association with the coral holobiont. Previous studies have demonstrated ubiquitous and conserved microbiota associated with some coral species (Bourne and Munn 2005; Rohwer et al. 2002) which play an active role in maintaining coral health and resilience (Rosenberg et al. 2007). For example, the mucus layer, skeleton and tissue of healthy corals all contain large populations of eukaryotic algae, bacteria and Archaea which confer a variety of benefits to their coral host, including photosynthesis,

the provision of nutrients and infection prevention (Ritchie 2006). Microbes are also particularly well suited to function as the holobiont's first responders to stressors because they encode a far greater variety of metabolic capabilities than do the coral or the zooxanthellae (Rohwer 2010).

There is increasing evidence that coral microbes are crucial in the biogeochemical cycling of the coral host with for example some coral-associates actively involved in nitrogen cycling processes, including nitrification, ammonium assimilation, ammonification and denitrification (Kellogg 2004; Kimes et al. 2010; Olson et al. 2009; Raina et al. 2009; Siboni et al. 2008; Wegley et al. 2007). Some bacteria hosted by corals have been identified to possess genes for nitrogen fixation (Olson et al. 2009), which is the biological conversion of gaseous nitrogen into ammonia. However only a few groups of bacteria are known to be capable of fixing nitrogen they play an important role in the cycling of nitrogen as many other organisms depend on these nitrogen fixers to meet their essential nitrogen needs. Called diazotrophic bacteria, they benefit the coral-symbiotic dinoflagellates at times when inorganic nitrogen supplies are limited (Lesser et al. 2007). This tight nutrient cycling between symbiotic partners is likely to enable the coral holobiont to flourish in the oligotrophic waters surrounding coral reefs.

1.4 Coral diseases

Despite their many benefits, certain microbes can cause coral bleaching and other diseases during conditions of environmental stress (Rosenberg et al. 2007). Unprecedented increase in coral disease has been documented in the Caribbean (Porter et al. 2001; Weil 2004; Weil et al. 2006; Weil et al. 2002), much less is known about the status of disease throughout the Indo-Pacific though. Preliminary surveys in Australia, the Philippines, Palau, and East Africa revealed significant and damaging new diseases in all locations surveyed (Harvell et al. 2007).

Since Black Band Disease (BBD) was reported as the first coral disease in 1973 (Antonius 1973), worldwide more than twenty nine distinct diseases have been described (Willis et al. 2004). However, following Koch's postulates[1], potential pathogens have been identified for only five of these diseases (Harvell et al. 2007).

Infectious diseases in coral can appear as lesions or distinct bands of tissue loss. Some coral diseases might be caused by a single organism (Kushmaro et al. 1996) while others involve a large and complex consortium of different microbes (Sekar et al. 2006). BBD for example is characterized by a combination of factors, including cyanobacterial biomass that creates an anoxic boundary and contributes to an increase in sulphide, which is toxic to underlying coral tissue. The causative agent of this complex polymicrobial disease has not been determined however (Sekar et al. 2006)

Both local (overfishing, nutrient enrichment) as well as global (climate change, ocean acidification) human-induced stressors seem to facilitate the emergence of coral diseases, with climate change and associated rising sea surface temperature most likely being a leading cause (Harvell et al. 2007). Temperature-induced coral bleaching increases the corals' susceptibility to more opportunistic pathogens and some pathogenic organisms seem to become more virulent in elevated temperature. Both bacteria, *Vibrio shiloi* and *Vibrio corallilyticus*, have been reported to cause bleaching in the corals *Oculina patagonica* and *Pocillopora damicornis*, respectively; however, the disease was only caused in stressed corals or when temperatures were elevated (Ben-Haim and Rosenberg 2002; Kushmaro et al. 1996).

A recently conducted metagenomics study revealed temperature elevation, nutrient addition and lowered pH as triggers for a dramatic increase in herpes-like viruses associated with corals

[1] A standard procedure to identify specific pathogens by Robert Koch, established in 1890.
Koch's postulates state: 1. The microbe must be found in all organisms suffering from the disease (and preferably not be present in healthy organisms). 2. The microbe must be isolated from the diseased organism and grown in pure culture. 3. The cultured microbe must cause the disease when introduced into a healthy organism. 4. The same microbe must be re-isolatable from the newly diseased organism (later addition, not by Koch).

(Vega-Thurber et al. 2009). It has been suggested that many coral diseases may not be caused by a specific pathogen but rather by opportunistic pathogens which are enhanced by environmental stressors (Lesser et al. 2007; Rohwer 2010). To clarify the coral disease phenomenon it is important however, to understand host–agent–environment interactions, pathology and factors that promote the virulence of the causative agent(s); only upon this achievement can rapid diagnosis be applied for better management of coral reef environments with response plans for specific outbreaks (Teplitski and Ritchie 2009). Until then, human activities that have been demonstrated to have direct effects on coral health (Bruno 2003; Carilli 2009; Garren et al. 2008; Smith 2008) need to be limited which is particularly important as climate continues to challenge corals (Bourne et al. 2009).

1.5 Study Sites: Ningaloo Reef and Rottnest Island

1.5.1 Ningaloo Reef

Part of this study was carried out in the Ningaloo Marine Park, Western Australia. With a total length of approximately 280 km Ningaloo Reef is the largest fringing coral reef system in Australia and the only extensive coral reef worldwide fringing the west coast of a continent (Taylor and Pearce 1999). The main reef line consists of a series of elongated reef segments which are punctuated by gaps with relatively deep channels through which the majority of lagoon flushing occurs. The shallow sedimentary lagoon has a mean depth of about 4 m with occasional patch and near-shore platform reefs. The close proximity of the reef to the coast varies from a few hundred metres to approximately 7 km and provides easy access from the shore. The central section of the 280 km long reef is only 4 km from the continental shelf, making it the closest point of the Australian mainland to the shelf break (Hearn and Parker 1988).

Two opposing currents, the pole-ward Leeuwin Current which meanders along the length of the western Australian (WA) coast and the localized equator-ward Ningaloo Current set up a series of counter-clockwise eddies which supply the reef system with recirculated nutrients, juvenile recruits, and fresh oceanic water (D'Adamo and Simpson 2001; Woo et al. 2006). The Ningaloo Current is likely to be associated with the introduction of nutrient-rich water off Ningaloo Reef through coastal upwelling and/or advection. The dynamic interaction between these two currents may redirect some of the water from the Ningaloo Current back towards the south and oceanographic modelling over the North West Shelf suggests that shelf currents connect the reef environments of Ningaloo Marine Park with those of the Dampier Archipelago/Cape Preston and Montebello/Barrow Islands. The average water temperature on Ningaloo Reef varies from 24^{o}C to 28^{o}C in summer whereas during the winter months the temperature ranges between 18^{o}C and 23^{o}C (Ceh et al. 2011).

1.5.2 Leeuwin Current

The Leeuwin Current (LC) is a warm, seasonally varying ocean current near the western coast of Australia with weaker southward flow during the austral summer when southerly winds are strongest. It originates near North West Cape (22°S) in Western Australia where it is mainly sourced by northern tropical Pacific Ocean water from the Indonesian Through-flow and the East Gyral Current (Wijjfels et al. 1992). This results in the atypically warm continental shelf waters of central and southern Western Australia which are warmer in winter and cooler in summer than the corresponding regions off the other continents, which are fed by solely cold equator-ward currents. The LC flows southward towards Cape Leeuwin (35°S) before turning eastwards into the Great Australian Bight. From south-eastern South Australia it is known as the South Australian Current and flows to north western Tasmania. The West Australian current and Southern Australian Counter Current, which are produced by the West Wind Drift on southern Indian Ocean and Tasmania,

respectively, flow in the opposite direction, producing one of the most interesting oceanic current systems in the world.

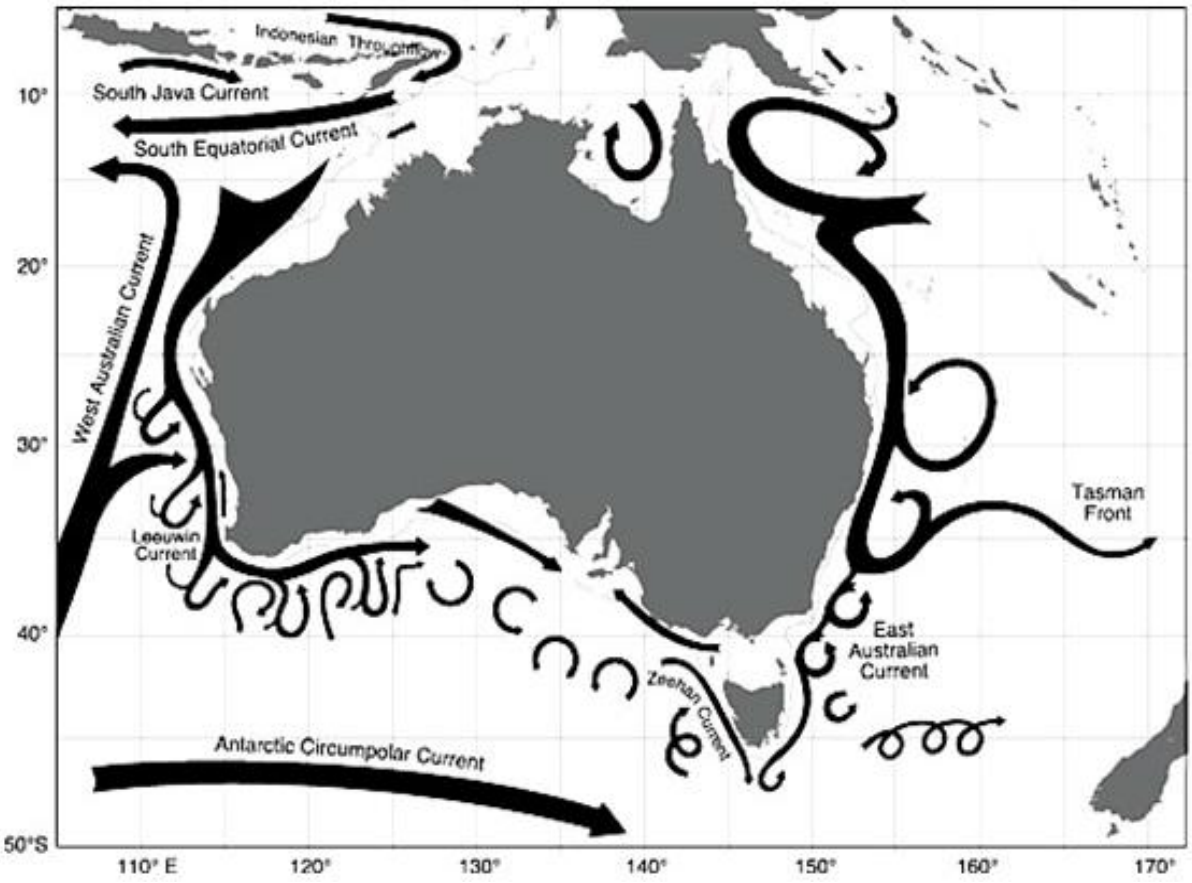

Fig. 1.1: A schematic representation of the major current systems in the Australian region (http://www.bom.gov.au/oceanography/forecasts/images/majorcurrents.jpgsource).
For further details see figure 2.1, chapter 2.

1.5.3 Rottnest Island

Rottnest Island (32°00'S, 115°31'E) is located 19 km off the coast from Fremantle and is 11 km long and 4.5 km wide at its widest point; its long axis is oriented east-west. During winter the island is warmed by the Leeuwin Current providing a higher minimum temperature (19°C) than the adjacent mainland coast (15°C). Due to this temperature regime Rottnest Island has the best developed coral communities of any area of the temperate coast with 25 species from 16 genera

represented (Veron and Marsh 1988). The two major effects of the LC on the coastal waters of Rottnest Island are the transport of tropical marine larvae from more northern latitudes, and increased winter water temperatures that enable many of these organisms to survive at Rottnest Island.

1.6 Motivation and Objectives

Coral microbiology is an emerging field, driven largely by a desire to understand, and ultimately prevent, the worldwide destruction of coral reefs (Rosenberg et al. 2007). Early coral microbial studies focussed on pathogens involved in coral diseases and relied on culturing the microbes of interest; however, only a small minority (<1%) of coral microbes can be cultured using standard procedures (Rohwer 2010).

The application of molecular and genomic techniques has revolutionized the field of environmental microbiology, opening a fascinating new avenue to a better understanding of coral-associated microbes and potential ways to study their functions within the coral holobiont. Analysis of conserved bacterial 16S rRNA genes has provided for example a far more complete picture of coral microbial community composition and helped identify novel organisms, new potential metabolic processes occurring within the holobiont along with potential causative agents of coral disease. However, the field of coral microbial ecology is still in its infancy and so is our knowledge about the multiple potential processes involving microorganisms in coral reefs.

With the exception of direct physical damage, the precise mechanisms of coral loss are still

unclear. Coral bleaching plays an important role, however, an obvious denominator in coral mortality is disease caused by epidemics from specific or opportunistic pathogens (Dinsdale et al.

2008). This highlights the necessity to understand microbial processes and their influence on coral reef ecosystem functioning and to elucidate the factors that are important in coral health.

The abundance of microorganisms around corals is well known; this dynamic microbiota has been shown to exist in several niches of corals, including the coral surface mucus layer (Bourne and Munn 2005), within coral tissue (Banin et al. 2001), the calcium carbonate skeleton (Koren and Rosenberg 2006) and in the water surrounding corals (Frias-Lopez et al. 2002). Moreover it is likely that within each of these habitats there are microniches colonized by different bacterial species (Ritchie and Smith 2004). Previous studies revealed a high coral specificity with coral-associated microbes. Microbial communities differ in composition between corals and their surrounding seawater, suggesting the association between the coral and its microbiota to be specific. Furthermore, similar bacterial populations seem to be associated with particular coral species (even if geographically separated), whereas different populations are found on different coral species (Rohwer et al. 2002; Rosenberg et al. 2007).

Various aspects of coral-microbial associations have been addressed and revealed valuable answers and new insights about our knowledge in coral microbiology. Most coral-microbial investigations have been focused on 'snap shot' studies though, rather than long term observations of a coral's life, and the structure of coral-microbial communities over time and space are largely unknown. Understanding microbial communities associated with corals and how they change through time is the key to understanding the health of reef corals (Bourne and Munn 2005). Continuous long-term records in coral-microbial partnerships observed in healthy coral reef systems are needed to provide a baseline and an overview of the 'normal ranges' in coral-microbial community structure in the intact coral holobiont. These long-term observations assist in characterizing reef responses to anomalous, environmental stressors and allow us to take measures reducing their causes to a sustainable level.

Rohwer and Kelley (Rohwer and Kelley 2004) suggested that corals may in fact harbour specific microbial communities for beneficial effect. Looking at the different stages in a coral's life and the varying needs in the different associated conditions, it seems likely that corals harbour microbial communities which change over time, depending on their present requirements in the respective life stage. The dynamics of microorganisms in different coral life stages, and particularly the establishment of the microbial-coral association and their role in early coral life stages, their function in coral settlement, recruitment and survival are not well understood and to date, few studies have addressed such questions.

Ningaloo Reef is a high-productivity, near pristine reef with relatively low human impacts; it is largely unaffected by land-based pollution and has so far shown unusual resistance towards the effects of climate change. With these conditions Ningaloo Reef provides unique opportunities to study coral-microbial interactions in a healthy coral reef system with major importance as a reference study site for future scenarios. Considering the importance of coral reefs, the challenges that are facing the coral holobiont in a continuously changing climate and the lack of sufficiently understanding the complex associations of the coral animal, this first coral-microbial study in Western Australia aimed to investigate the dynamics of coral-associated microbial communities in a near-pristine coral reef ecosystem to better understand seasonal, geographical and coral-life-cycle related variations in coral-microbial communities.

CHAPTER 2

Coral–associated bacterial communities on Ningaloo Reef, Western Australia

This work has been published in FEMS Microbiology Ecology:

Ceh J, van Keulen M, Bourne DG (2011) Coral-associated bacterial communities on Ningaloo Reef, Western Australia. FEMS Microbiology Ecology 75: 134–144

2.1 Abstract

Coral-associated microbial communities from three coral species (*Pocillopora damicornis*, *Acropora tenuis* and *Favites abdita*) were examined every three months (January, March, June, October) over a period of one year on Ningaloo Reef, Western Australia. Tissue from corals was collected throughout the year and additional sampling of coral mucus and seawater samples was performed in January. Tissue samples were also obtained in October from *P. damicornis* coral colonies on Rottnest Island off Perth, 1200 km south of Ningaloo Reef to provide comparisons between coral-microbial associates in different locations. The community structure of the coral-associated microorganisms were analysed by phylogenetic analysis of 16S rRNA gene clone libraries which demonstrated highly diverse microbial profiles among all coral species sampled. Principal component analysis (PCA) revealed that samples grouped according to time rather than species indicating that coral-microbial associations may be a result of environmental drivers such as oceanographic characteristics, benthic community structure and temperature. Tissue samples from *P. damicornis* at Rottnest Island revealed similarities in bacteria to the samples at Ningaloo Reef. This study highlights that coral-associated microbial communities are highly diverse, however, the complex interactions that determine the stability of these associations are not necessarily dependant on coral host specificity.

2.2 Introduction

Coral reefs worldwide are currently subjected to unprecedented degradation, likely caused by anthropogenic influences including increased sea surface temperatures, coastal degradation, pollution, diseases, ecosystem imbalance and the synergistic effects of multiple stressors (Bruno et al. 2007; Harvell et al. 2002; Pandolfi et al. 2003). These changing anthropogenic stressors often upset the delicate balance between the coral animal and its associated microorganisms, which includes symbiotic dinoflagellates (Zooxanthellae), endolithic algae, fungi, Bacteria, Archaea and viruses, all closely associated with the coral animal and together representing the dynamic assemblage referred to as the coral holobiont (Bourne et al. 2009; Rohwer et al. 2002; Rosenberg et al. 2007). The importance of coral-microbial interactions is increasingly being recognized with studies demonstrating the active role these associated microbial communities have in maintaining coral health and resilience. Bacteria associated with corals have been studied extensively and found to occupy several micro niches within the coral host including the surface mucus layer, the tissue and the calcium carbonate skeleton (Littman et al. 2009; Ritchie and Smith 1997; Rohwer et al. 2001; Rohwer et al. 2002; Rosenberg et al. 2007). Information on the spatial and temporal composition of bacterial communities associated with corals is also accumulating with previous studies suggesting some associations may be relatively stable and species specific (Rohwer et al. 2002). The functional role of these bacteria associated with coral is poorly understood although reports have indicated they may be involved in the provision of nutrients (Olson et al. 2009; Shashar et al. 1994) and exclusion of potentially pathogenic microorganisms (Ritchie 2006).

Corals are increasingly faced with changing conditions on both regional and global scales. Understanding microbial communities associated with corals, their functional roles and how they change through time is the key to understanding how these changes will affect the coral holobiont. Shifts in the bacterial community composition may affect coral health and increase the

susceptibility of the host to disease (Bourne and Munn 2005; Bourne et al. 2008; Ritchie 2006). The specificity of coral-bacterial associations is still not well understood and there is a requirement for long term monitoring studies to enhance our understanding of the interactions between the coral host and associated microbes, and to assess the potential influence of environmental parameters structuring these communities. If bacteria routinely form species-specific associations with corals, it would be expected that such associations would be maintained over space and time (Rohwer et al. 2002). However, several investigators have shown that the bacterial population associated with a coral can change as a function of disease or varying environmental conditions (Bourne et al. 2008; Hong et al. 2009; Koren and Rosenberg 2006; Pantos et al. 2003; Ritchie and Smith 2004). Based on these and other studies Reshef and colleagues (Reshef et al. 2006) proposed the Coral Probiotic Hypothesis suggesting that corals can adapt rapidly to changing environmental conditions by altering their population of symbiotic bacteria. To study coral microbial interactions it is necessary to determine which microbes reside on or within corals, whether there is a geographical consistency and how the dynamics of the coral-associated consortia vary in time. Multiple impacts, like geographical and environmental factors (Bourne et al. 2008; Hong et al. 2009; Koren and Rosenberg 2006; Littman et al. 2010) as well as coral physiology (Littman et al. 2010) have been suggested to drive the structure of coral-associated microbes.

This study examines coral-microbial associations over time for three coral species, *Pocillopora damicornis, Acropora tenuis* and *Favites abdita*, living in close proximity to each other on Ningaloo Reef. Corals were sampled repeatedly throughout the year to investigate whether temporal environmental changes would lead to natural variation in coral-associated bacterial diversity. To test whether the growth form of corals play a role in structuring bacterial consortia as has been suggested previously (Sunagawa et al. 2010) we included branching (*P. damicornis, A. tenuis*) versus massive corals (*F. abdita*) in our study. *P. damicornis* samples were collected from two locations on the Western Australian coast, Ningaloo Reef and Rottnest Island, to identify which

bacteria might be conserved across geographically distinct locations. This study provides the first analysis and comparison of microbial communities from Western Australian corals. Compared to other reef systems, warm water bleaching events and outbreaks of coral diseases are virtually unknown for Ningaloo Reef, which makes it an excellent reference study place representing a relatively healthy coral reef environment.

2.3 Materials and Methods

2.3.1 Sampling sites

Ningaloo Reef is the largest fringing coral reef system in Australia (280 km in length) and the only extensive coral reef worldwide fringing the west coast of a continent (Taylor and Pearce 1999). The main reef line consists of a series of elongated reef segments which are punctuated by gaps with channels through which the majority of lagoon flushing occurs. The shallow sedimentary lagoon has a main depth of about 2m with occasional patch and nearshore platform reefs.

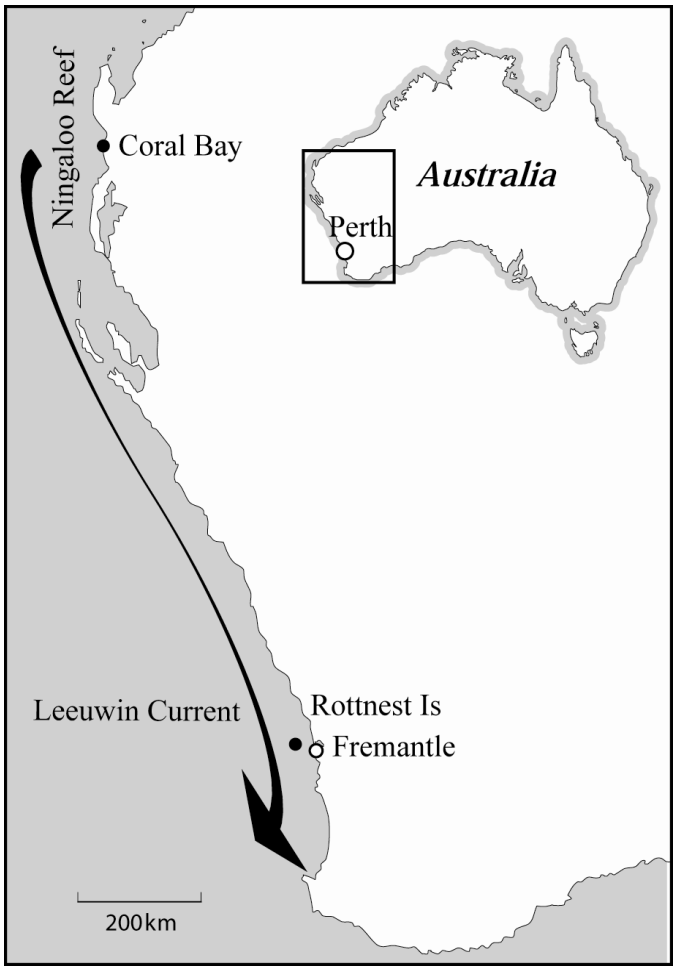

Figure 2.1: Location of study sites (Coral Bay and Rottnest Island) where corals were sampled. The polewards flowing Leeuwin Current indicated by the arrow is part of a complex interplay current system that moves seawater along the Western Australian coast and is only represented as a simplification in this figure.

Rottnest Island (32°00'S, 115°31'E) is located 19 km off the coast from Fremantle and is 11km long and 4.5 km wide at its widest point; its long axis is oriented east-west. During winter the island is warmed by the Leeuwin current (Figure 2.1) providing a higher minimum temperature (19°C) than the adjacent mainland coast (15°C). This temperature regime allows 25 species of 16 genera of corals to occur around Rottnest Island which makes it the best developed coral community of the temperate coast (Veron and Marsh 1988).

2.3.2 Sample collection

Two replicate coral colonies, similar in size, of three coral species, *Pocillopora damicornis*, *Acropora tenuis* and *Favites abdita*, were tagged on a reef flat (5-6m water depth) near Coral Bay (23°07'S, 113°07'E), Ningaloo Reef, Western Australia. Two replicate coral nubbins (approximately 2 cm in size) from each tagged colony were placed in individual zip-lock bags under water, rinsed on the surface twice with 0.2 µm filtered and autoclaved artificial seawater (ASW) to remove loosely attached microbes, and then placed on ice. Massive corals were sampled using hammer and chisel to extract a 2 x 2 cm piece of coral. The coral samples were air brushed with 2 mL of ASW to remove the coral tissue including the associated microorganisms from the coral skeleton. The tissue slurry was aliquoted into cryovials and stored at -80°C until required for analysis. Two replicate coral mucus samples per coral colony were collected with 50 mL syringes. Seawater samples were collected in sterile plastic bottles (1L) about 2m distance from each tagged coral colony. Mucus samples as well as water samples were filtered through Sterivex (0.22 µm) filter columns (Millipore) and stored at -80°C for later analysis. Samples were processed within one hour of sampling. Sampling scars healed before re-sampling and all corals appeared to be healthy throughout the sampling period and six months after the last collection. Samples were collected randomly from the coral colonies, regardless of former samplings. Coral tissue, coral mucus and water samples were collected in January to assess bacterial communities between these different

environments, which have been shown to differ in previous studies (Bourne and Munn 2005; Frias-Lopez et al. 2002; Pantos et al. 2003; Rohwer et al. 2002). Subsequent sampling in March, June and October 2008 from Ningaloo Reef was of coral tissue only. Seawater temperatures in close proximity to the sampling site on Ningaloo Reef were recorded at 30 min intervals using a temperature logger (Odyssey, Temperature Recorder) for the sampling period from late March to November to assess possible correlations of changes in coral microbiota with seawater temperature. A logger fault resulted in the loss in temperature data from January till March, with the missing information substituted with sea surface temperature data of Ningaloo Reef from the National Oceanic Atmospheric Administration (NOAA) Satellite and Information service (http://coralreefwatch.noaa.gov/satellite/current/sst_series_ningaloo_cur.html).

Additional replicate tissue samples from two *P. damicornis* colonies were sampled once from Rottnest Island (32°00'S, 115°31'E), 1200 km south of Coral Bay to allow for comparisons between associated bacteria from geographically dispersed corals of the same species.

2.3.3 DNA extraction and purification

Coral tissue slurries (2 mL) were centrifuged at 13000g to pellet the tissue and the associated microbiota. The supernatant was removed and the pellet re-suspended in 500 µl of buffer (0.75 M Sucrose, 40 mM EDTA, 50 mM Tris base, pH 8.3). Total DNA was extracted, following the methods specified in Bourne et al., (Bourne et al. 2008). For extraction of DNA from mucus and seawater samples the Sterivex filter units were filled with 1.6 mL of lysis buffer (0.75 M sucrose, 40 mM EDTA, 50 mM Tris base, pH 8.3) and 0.2 mL lysozyme solution (1mg mL-1) added. The filter unit was incubated at 37°C for one h, 0.2 mL Proteinase K/sodium dodecyl sulphate (SDS) solution (0.2 µg mL-1 Proteinase K + 1% SDS) added and the filter column incubated at 55°C for 1 h. The lysate was recovered, the Sterivex filter columns rinsed with an additional 1mL lysis buffer and the pooled lysates extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol

(25:24:1, pH 8). The liquid phase was removed and extracted with an equal volume of chloroform-isoamyl alcohol (24:1); after removing the aqueous phase, 0.1 mL sodium acetate (3M, pH 5.2) along with 1 mL 100% isopropanol were added and the DNA pelleted at 13000 g at 4°C for 10 min and washed with 70% ethanol (modified method by Schauer (Schauer et al. 2000)). Extracted total DNA of all samples (coral tissue, coral mucus and seawater) were re-suspended in 30 µl of sterile Milli-Q water, loaded on a 1.2% low-melting agarose gel and the high quality DNA (>2 kb) cut out and purified with a QIAquick gel extraction kit (QIAGEN) following the instructions of the manufacturer. The DNA was recovered from the columns in two washes (30µl and 20 µl) of sterile Milli-Q water and stored at -20°C until further processing.

2.3.4 PCR amplification of 16S RNA ribosomal genes and clone library construction

Bacterial specific primers 63f and 1387r (Marchesi et al. 1998) were used to amplify the 16S rRNA genes from extracted DNA for bacterial clone library construction of coral tissue, coral mucus and seawater. The PCR mixtures (50 µl) contained 0.2 pmol µl-1 of each primer, 200 mM each deoxynucleoside triphosphate, 1X PCR buffer (Tris-Cl, KCl, (NH4)2SO4, 1.5 mM MgCl2), 0.08% (w/v) bovine serum albumin and 1.25 U of Taq polymerase (Scientifix, Clayton, VIC, Australia). PCR was performed with an Applied Biosystems 2720 thermocycler and programmed with an initial 4 min step at 94°C and 30 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min and a final extension for 10 min at 72°C.

The amplified bacterial DNA of pooled replicates for each extracted sample was ligated into the TOPO-TA cloning vector (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. This resulted in 17 clone libraries with ligations which were then submitted to the Australian Genome Research Facility for transformation, cloning and subsequent sequencing. Ninety-six clones were sequenced from each library using the M13f primer. The nucleotide

sequence data of all clones reported in this paper appear in the GenBank nucleotide sequence database under the accession numbers GU184380 - GU185837.

2.3.5 Sequence analysis and statistical analysis

Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al. 1996) Sequence data were aligned to the closest relative using the BLAST database algorithm (Altschul et al. 1997). Sequence affiliations were determined by >97% identity to bacterial 16S rRNA gene sequences in the GenBank database. From a total of 1450 16S rRNA gene sequences bacteria were grouped into operational taxonomic units (OTUs) based on the assumption that bacteria which share greater than 97% sequence identity represent an individual OTU (Ward 1998).

The variation of microbial diversity in the clone libraries was investigated by using the following indices and models (Magurran 1988): The *Shannon Diversity Index* (H) (Shannon and Weaver 1963), the *Simpson's evenness index* (D) (Simpson 1949), *Fisher's Alpha* log series richness index and coverage (C) values (Good 1953). A principal components analysis (PCA) was implemented to determine if bacterial profiles from coral samples grouped according to species or time. Any ribotype constituting 5% or more (arbitrarily assigned as dominant) of each clone library was included in the principal components analysis. PCA statistical analyses were carried out using PAST statistical software (Ryan et al. 1995).

2.4 Results

Bacterial clone libraries derived from three coral species *P. damicornis*, *A. tenuis* and *F. abdita* collected in January from Ningaloo Reef were dominated by *Alphaproteobacteria* and *Gammaproteobacteria* affiliated sequences (Table 2.1). *Alphaproteobacteria* sequences were dominant in *P. damicornis* tissue (73%) although they only represented 15.9% and 17.3 % of *A. tenuis* and *F. abdita* libraries respectively. *Gammaproteobacteria* sequences constituted the majority of clones in *A. tenuis* (72%) and *F. abdita* (34.7%) libraries although they were in low relative abundance in *P. damicornis* libraries (5.4%). In addition *P. damicornis* contained a relatively high number of *Flavobacteria* (6.5%) whereas 8% *Betaproteobacteria* and 8% *Actinobacteria* affiliated sequences were obtained from the coral *F. abdita*. Additional clone libraries constructed from the mucus derived from the same coral colonies displayed a higher bacterial diversity as assessed by diversity indices including *Shannon Diversity Index* (H), *Simpson's evenness index* (D) and *Fisher's Alpha* (Fisher et al. 1943) log series richness index (Table 2.2). Similar to the tissue samples, *Alphaproteobacteria* and *Gammaproteobacteria* affiliated sequences dominated these mucus libraries however *Bacteroidetes* and *Flavobacteria* affiliated sequences were also more predominant, constituting between 6.5 to 12% and 10.6 to 19.6% of the libraries respectively.

The sequences obtained from the surrounding seawater demonstrated a high resemblance to the mucus libraries with the relative abundance of retrieved *Bacteroidetes* and *Flavobacteria* affiliated sequences being similar. The exception was that *Alphaproteobacteria* affiliated sequences were more often associated with coral mucus (two to three fold) than with seawater, whereas the abundance of *Gammaproteobacteria* was much higher (two to four fold) in seawater libraries compared to coral mucus libraries (Table 2.1).

The bacterial profiles associated with the coral tissue changed over time as the same coral colonies were repeatedly sampled in January, March, June and October. Although

Alphaproteobacteria and *Gammaproteobacteria* affiliated sequences still dominated the libraries, their relative proportions changed through the sampling period. For example, compared to tissue samples in January tissues in March demonstrated a decrease in both *Alphaproteobacteria* and *Gammaproteobacteria* affiliated sequences while ribotypes affiliated with *Betaproteobacteria*, *Bacteroidetes* and *Flavobacteria* were more prominent in March (Table 2.1). Coral microbial profiles derived from June samples demonstrated the most drastic shift with *Gammaproteobacteria* sequences dominating in all libraries (between 47.4 to 73.4%). *Alphaproteobacterial* sequences only constituted between 2.1 to 13.4% of the libraries while, *Bacillales* affiliated sequences represented 8.4 to 13.8% of the libraries. Profiles of October derived samples again shifted and like January and March samples displayed higher relative abundance of *Alphaproteobacteria* affiliated sequences (39.1 to 44.4%) while the relative abundance of *Gammaproteobacteria* sequences declined (9.9 to 14.9%). No *Bacillales* affiliated sequences were retrieved while small numbers of *Bacteroidetes* (2.3 to 5.4%), *Flavobacteria* (0 to 6.5%) and *Cyanobacteria* (3.3 to 9.2%) were retrieved from all coral species (Table 2.1).

Libraries constructed from the tissue of *P. damicornis* coral sampled from geographical distant colonies at Rottnest Island were pooled since the relative abundance of ribotypes was consistent. Coral-associated bacterial profiles at each site displayed a strong similarity in diversity and community structure (Figure 2.2, Table 2.2). Both Ningaloo and Rottnest libraries were dominated by

Table 2.1. Proportions of bacterial taxonomic classes for each clone library

Bacteria classification	January clone libraries							March clone libraries				June clone libraries				October clone libraries				
	P. damicornis	*A. tenuis*	*F. abdita*	*P. damicornis M*	*A. tenuis M*	*F. abdita M*	Sea Water	*P. damicornis*	*A. tenuis*	*F. abdita*	*P. damicornis*	*A. tenuis*	*F. abdita*	*P. damicornis*	*A. tenuis*	*F. abdita*	*P. damicornis R*
Alphaproteobacteria	73.1%	15.9%	17.3%	30.4%	21.3%	21.7%	9.6%	25.0%	11.0%	23.3%	3.2%	2.1%	13.7%	44.4%	27.6%	39.1%	44.1%
Betaproteobacteria	2.2%	2.3%	8.0%	0.0%	0.0%	1.1%	0.0%	5.7%	21.9%	8.1%	2.1%	1.1%	8.4%	0.0%	2.3%	1.1%	3.2%
Gammaproteobacteria	5.4%	72.7%	34.7%	9.8%	9.6%	26.1%	47.0%	25.0%	17.8%	18.6%	73.4%	71.6%	47.4%	9.9%	14.9%	13.0%	23.7%
Deltaproteobacteria	0.0%	0.0%	1.3%	2.2%	1.1%	0.0%	0.0%	0.0%	0.0%	2.3%	0.0%	0.0%	0.0%	2.5%	1.1%	1.1%	6.1%
Bacteroidetes	2.2%	0.0%	1.3%	6.5%	10.6%	12.0%	6.0%	4.5%	4.1%	4.7%	0.0%	0.0%	0.0%	3.7%	2.3%	5.4%	3.1%
Flavobacteria	6.5%	1.1%	2.7%	18.5%	10.6%	19.6%	16.9%	5.7%	2.7%	8.1%	0.0%	0.0%	0.0%	0.0%	5.7%	6.5%	0.8%
Sphingobacteria	0.0%	1.1%	0.0%	0.0%	1.1%	1.1%	2.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Actinobacteria	0.0%	1.1%	8.0%	0.0%	0.0%	0.0%	0.0%	4.5%	2.7%	0.0%	1.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacilli	0.0%	0.0%	2.7%	0.0%	0.0%	1.1%	2.4%	0.0%	0.0%	0.0%	13.8%	8.4%	11.6%	0.0%	0.0%	0.0%	0.0%
Cyanobacteria	0.0%	0.0%	0.0%	2.2%	0.0%	0.0%	0.0%	0.0%	2.7%	0.0%	0.0%	0.0%	1.1%	6.2%	4.6%	3.3%	3.1%
Firmicutes	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.2%	0.0%	0.0%	0.0%	1.2%	0.0%	0.0%	0.0%
Unclassified	10.8%	5.7%	24.0%	30.4%	45.7%	17.4%	15.7%	29.5%	37.0%	33.7%	6.4%	16.8%	17.9%	32.1%	41.4%	30.4%	15.9%

* M=mucus derived libraries

Alphaproteobacteria (44.4 and 44.1%) and *Gammaproteobacteria* (9.9 and 23.7%) respectively (Figure 2.2). In addition, sequences related to *Deltaproteobacteria* (2.5 and 6.1%), *Bacteroidetes* bacteria (3.7 and 3.1%) and *Cyanobacteria* (6.2 and 3.1%) were retrieved. The Ningaloo clone library additionally contained a small proportion of *Firmicutes* related sequences while *Betaproteobacteria* and *Flavobacteria* sequences were retrieved from tissue of corals from Rottnest Island.

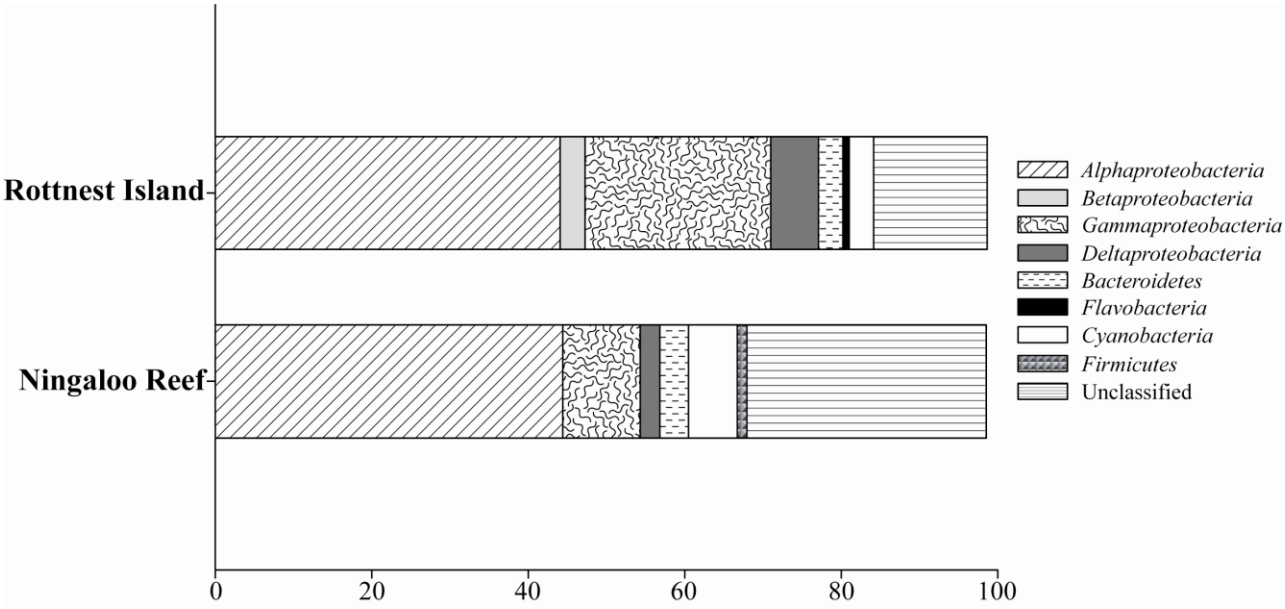

Figure 2.2: Dominant bacterial 16S rRNA gene sequence affiliations for *P. damicornis* clone libraries in October. Sequences were grouped into dominant ribotypes (>5% of clone libraries) at the phylum and class level.

Sequence affiliations at the lower taxonomic levels of genus and family were incorporated into a principle component analysis (PCA) which, similarly to the grouping at the class and phylum level, demonstrated that libraries derived from the corals grouped according to sampling time and not species type (Figure 2.3). For example, January samples were correlated with an increased relative abundance of *Alcanivorax sp.*, *Methylarcula sp*. and *Salinivibrio sp*. affiliated sequences.

In comparison, March coral tissue libraries correlated with *Achromobacter sp., Acinetobacter sp*., *Bacteroidetes*, and *Brevundimonas sp*.. June libraries grouped based on strong correlations to *Bacillales sp*. and *Vibrio sp*. whereas the clone libraries derived from October sampling associated with *Cyanobacteria, Erythrobacter sp*. and *Rhodobacter sp*. sequences. Interestingly, the October clone library of *P. damicornis* collected from Rottnest Island grouped closely to the clone libraries derived from the coral samples collected in October on Ningaloo Reef, showing a strong consistency within seasonal bacterial communities regardless of geographical separation (Figure 2.3).

Coral mucus samples collected in January grouped together based on correlations with *Flavobacteria*, *Litoricola sp.*, *Methylarcula sp*. and *Pseudoalteromonas sp*. sequences, although this was also consistent with the seawater clone library. A comparison between clone libraries showed that none of the dominant OTU groups (ribotypes >5% within each library) were consistently found in the libraries derived from the same coral in January and June clone libraries. In addition, OTU groups that did occur at more than one time point were generally detected in all coral species. Comparison of diversity indices between all libraries revealed a similar bacterial diversity for different coral species for most sampling times (Table 2.2). Based on the total number of OTUs, the highest diversity in bacteria was found in March and October, which represent the months with the highest and lowest water temperature throughout the sampling period. The water temperatures on the actual sampling days was (28.8°C) and (21.8°C) respectively (Supplementary figure S.2.1).

Table 2.2: Diversity indices calculated from OTU's of 16S rRNA gene clones derived from all clone libraries.

Parameters	January clone libraries							March clone libraries				June clone libraries				October clone libraries			
	P. damicornis	*A. tenuis*	*F. abdita*	*P. damicornis M*	*A. tenuis M*	*F. abdita M*	Seawater	*P. damicornis*	*A. tenuis*	*F. abdita*		*P. damicornis*	*A. tenuis*	*F. abdita*		*P. damicornis*	*A. tenuis*	*F. abdita*	*P. damicornis R*
No. of clones analysed	93	88	75	92	94	92	83	88	73	86		94	95	95		81	87	92	65
No. of OTU groups (ribotypes)	8	7	8	6	6	8	7	9	10	8		3	4	6		6	9	8	8
Coverage of clone libraries (%)	35.48	80.68	48	32.61	29.79	58.7	60.24	34.09	39.73	40.7		87.23	82.11	67.37		28.4	32.18	29.35	26.15
Shannon diversity *(H')*	1.47	1.08	1.45	1.47	1.51	1.71	1.58	2.13	2.05	1.99		0.55	0.51	1.13		1.73	1.9	1.87	1.99
Fisher's alpha ()	3.36	1.93	3.19	2.26	2.34	2.6	2.22	4.36	5.4	3.24		0.61	0.89	1.62		2.64	4.59	3.84	5.9
Simpson's evenness *(D)*	0.66	0.51	0.66	0.71	0.74	0.78	0.76	0.88	0.84	0.85		0.31	0.25	0.55		0.81	0.81	0.83	0.79

* M=mucus derived libraries

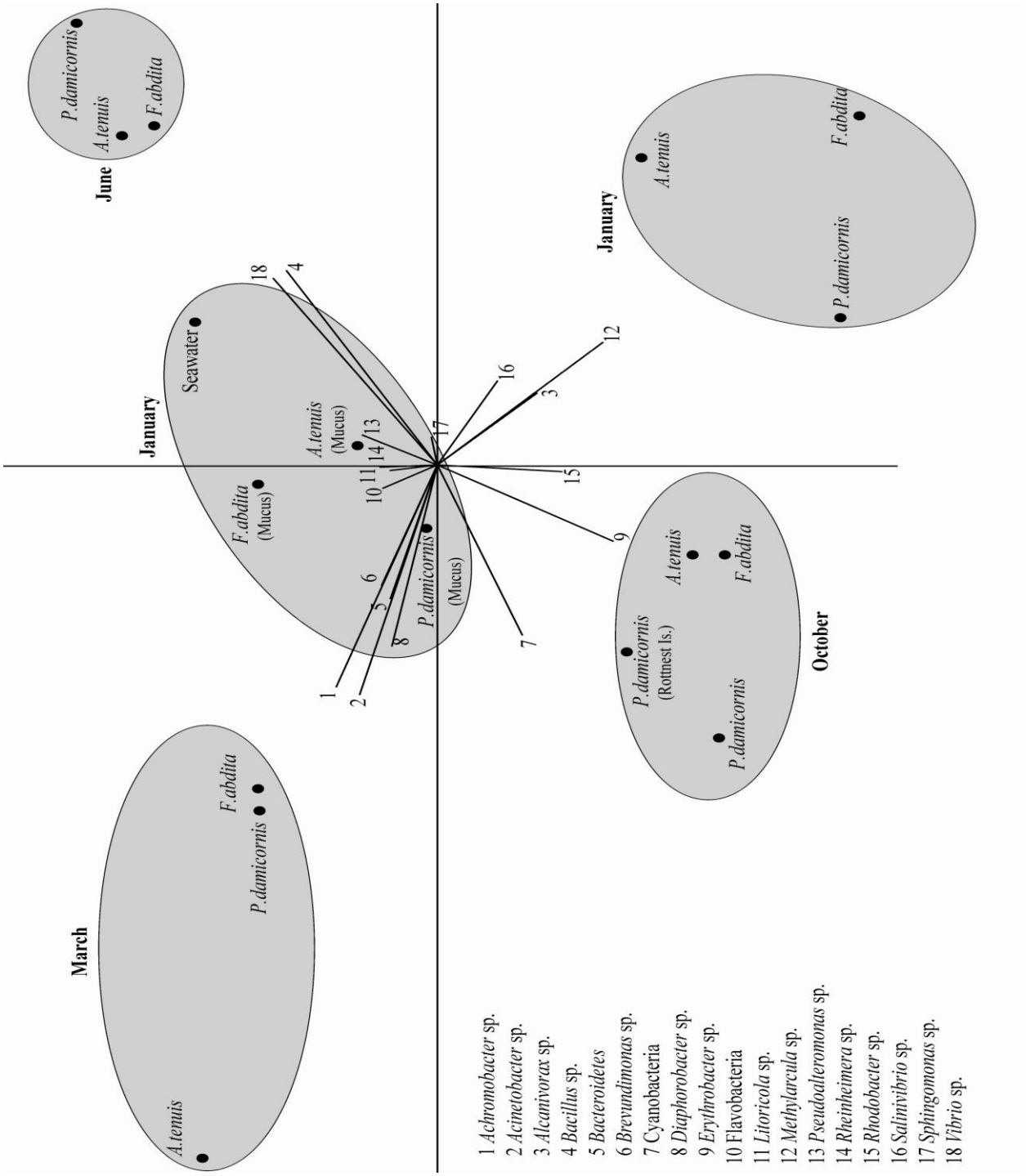

Figure 2.3: Biplot principal component analysis of clone library 16S rRNA gene sequences (family and genus level). Black lines are vectors representative of dominant OTUs (>5% of one or more libraries) driving the differences between clone libraries .Sequence affiliations are included in the analysis and represented by the numbers on the vector coordinates.

2.5 Discussion

Previous studies using molecular techniques to profile the bacterial 16S rRNA genes associated with corals have demonstrated highly diverse and abundant microbial communities (Bourne and Munn 2005; Cooney et al. 2002; Frias-Lopez et al. 2002; Rohwer et al. 2001; Rohwer et al. 2002). For example Rohwer and colleagues (Rohwer et al. 2001) statistically estimated as many as 6000 different ribotypes to be associated with corals. Despite this high diversity in the coral microbiota, a conserved microbial community has been suggested in some coral species (Bourne et al. 2008; Frias-Lopez et al. 2002; Ritchie and Smith 1997; Rohwer et al. 2002) and bacterial populations from the same coral species have been shown to be consistent even if coral individuals are separated spatially or temporally (Rohwer et al. 2002). Results from this study similarly demonstrate high bacterial diversity associated with corals from the Western Australian coast. However, sequence affiliations at the taxonomic levels of class and phylum, as well as at the genus and family level, demonstrated that libraries derived from three physically adjacent coral species sampled at four different time points throughout the year grouped according to sampling time and not coral species. These conclusions were drawn from consistent observations in dominant bacterial ribotypes (>5% of libraries) recovered from clone libraries of each coral species at each sampling time and suggest that while different coral species may harbour similar bacterial communities there is low species-specificity in the coral bacterial associations.

Corals and their associated bacteria exist in a delicate balance that is crucial to their survival, and the maintenance of homeostasis has been suggested as a critical factor to the persistence of a mutualistic symbiosis (Kline 2004). In this study, bacterial profiles for continuously sampled corals were highly different for each sampling time, which strongly indicates temporal shifts in bacterial communities within these coral species at this site.

The results do not suggest major long term homeostasis between the coral host and its' associated bacteria. A recent study by Hong and colleagues (Hong et al. 2009) investigated the species specificity of bacteria associated with the coral *Stylophora pistillata* and suggested that multiple dynamic factors including seasonal and geographic features were drivers for bacterial diversity in individual coral colonies, supporting the results in this current study. Hong and colleagues (Hong et al. 2009) showed little bacterial species specificity for the *Stylophora* colonies under investigation, however, speculated that such species specificity maybe variable for different corals. Their analysis of previous studies on coral-associated bacteria also showed that spatial and temporal factors were important in shaping these associations though distinct species specific bacterial profiles were identified in some Caribbean corals. For example, the Caribbean coral species *Montastraea annularis* and *Diploria strigosa* were found to harbour similar microbial consortia, with over half of the associated bacteria being present in both coral species (Klaus et al. 2005). However, a study by Rohwer and colleagues (Rohwer et al. 2002) noted that bacterial communities associated with healthy colonies of *D. strigosa* differed substantially from those reported from the same coral species in a study by Frias-Lopez and colleagues (Frias-Lopez et al. 2002) and highlighted a different methodology as a possible explanation for these differences. A comparison of two studies, which both investigated the diversity of bacteria associated with the coral *M. franksi* (Rohwer et al. 2001; Rohwer et al. 2002), indicated no essential overlap between retrieved 16S rDNA sequences either (Rohwer et al. 2002). All these studies together, (including the current results) indicate ubiquitous and generally conserved microbial-coral consortia but don't necessarily suggest that different coral species harbour distinct bacterial communities.

Most studies to date investigating coral-bacterial association have used clone libraries to assess bacterial diversity which due to inherent limitations under-sample the overall diversity of bacteria associated with corals and may contribute to the lack of overlap in sequences retrieved within and between studies. Developing sequencing technologies that are able to provide in-depth

analysis of bacterial diversity can overcome these limitations. A recent study using deep sequencing and analysis of >350.000 16S rRNA gene tags demonstrated that bacterial community composition displayed similar profiles among closely related coral in the same genus or family, but not at higher coral taxonomic levels (Sunagawa et al. 2010). Applying these developing in-depth sequencing technologies to analyse temporal changes and anthropogenic effects will further enhance our understanding of the stability of coral bacterial associations. Sunagawa and colleagues (Sunagawa et al. 2010) also highlighted the possibility that morphology may play a role in structuring coral microbiota. Our current study investigated three coral species from three different families (Acroporidae, Pocilloporidae and Faviidae) and though all coral demonstrated similar coral-associated profiles at the same time, this was independent of the coral morphology which included both branching and massive forms.

Given the assumption that microbial communities live in beneficial relationships with their coral host and that corals are exposed to various environmental factors throughout a reproductive cycle, it makes sense that corals harbour different bacterial types at different times according to the benefit these bacteria can provide to the present requirements of their host. Koren and Rosenberg (Koren and Rosenberg 2006) reported dynamic bacterial communities associated with healthy *Oculina patagonica* corals and identified temperature as one factor causing microbial shifts between seasons. While *Vibrio splendidus* affiliated sequences were dominant (35% of sequenced clones) and appeared stable in both summer and winter, the next ten most abundant clusters of bacteria differed between seasons. Results from our study detected neither a dominant group of bacteria throughout the year nor a bacterial class to be dominant at all times. Corals in January (with the exception of *P. damicornis*), were dominated by *Gammaproteobacteria* while the relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* sequences were similar in March samples. A shift towards *Gammaproteobacteria* (47.4 to 73.4%) was observed in all June samples with sequences related to *Vibrio* species being largely responsible for this change representing most

of the *Gammaproteobacteria* retrieved sequences in these samples. *Bacillales* related sequences also increased in the June samples and represented between 8.4 to 13.8% of sequences in the clone libraries (Table 2.1). At the final sampling time (October), *Alphaproteobacteria* related sequences were again dominant. Some classes of bacteria were mainly or exclusively found in particular months. For example *Bacillales* affiliated sequences were highly abundant in June from all coral derived libraries, whereas *Bacteroidetes* as well as *Flavobacteria* sequences were detected in most coral libraries throughout the sampling period, except in June. *Deltaproteobacteria* and *Cyanobacteria* were abundant in October. Despite *Vibrio* species being previously implicated in bleaching of some coral species, the shift in June (Winter) to a *Vibrio* dominated community did not visibly compromise coral health which supports previous findings that members of this group may form a natural part of the microbial population of healthy corals (Bourne and Munn 2005). These bacteria are likely involved in important roles within the coral host including fixation of nitrogen (Olson et al. 2009), which may be particularly important in times of nutrient limitations such as winter months in oligotrophic environments.

Mucus-associated microbial communities are taxonomically and functionally diverse (Bourne and Munn 2005; Ritchie 2006) and differ from the bacterial populations in coral tissue and the surrounding seawater (Koren and Rosenberg 2006). In this study, mucus from three different coral species contained similar bacterial communities and all mucus samples clustered closely on a PCA plot along with the seawater sample. Despite clustering of mucus and water samples, *Alphaproteobacteria* affiliated sequences were more abundant in coral mucus than in seawater, whereas *Gammaproteobacteria* were higher in seawater libraries compared to coral mucus. The number of *Bacteroidetes* and *Flavobacteria* affiliated sequences however was found to be very similar in both mucus and seawater libraries. This potentially indicates that coral mucus selects for *Alphaproteobacteria* rather than *Gammaproteobacteria* when bacteria of both classes are abundant and available in the seawater column. The structure of bacterial communities within coral mucus is

controlled by specific receptors which bind bacteria on the coral surface mucus (Kvennefors et al. 2008) and by signals and biocides found in coral mucus (Brown and Bythell 2005; Ritchie 2006; Shnit-Orland and Kushmaro 2009). Given the fact that the coral mucus layer provides a substrate for microbial growth (Ducklow and Mitchell 1979; Herndl and Velimirov 1986; Rublee et al. 1980) and interactions between the coral host and bacteria are initiated during the colonization of this layer, the mucus might serve as a selection barrier for bacteria which enter into close and stable associations within the coral host tissue. Bacteria which do not colonise this niche potentially only remain in the coral mucus temporarily and are removed by the cleansing mechanism of sloughing subsequently initiating a new selection cycle.

Coral reefs are known for low nutrient concentrations (Gast et al. 1999; Gili and Coma 1998; Muscatine 1980; Rahav et al. 1989; Szmant et al. 1990); increased algal abundance can cause elevated levels of DOC due to excess photosynthate released into the water column by the algal communities (Smith et al. 2006). Once algae become highly abundant and algal tissue begins decomposing, even larger amounts of organic carbon can be released into the water column and utilized by heterotrophic bacteria (Dinsdale et al. 2008). Previous studies have shown that elevated DOC levels can disrupt the balance between the coral and its associated microbiota by accelerating the growth rate of bacteria living in the coral mucus layer (Kline 2004; Smith et al. 2006). Algal outbreaks with a dominant abundance of *Sargassum* and *Turbinaria* occur seasonally on Ningaloo Reef (pers. com. van Keulen), starting in October/November, peaking around March and dying off in May/June. The seasonal pattern of high productivity and abundance of algae on Ningaloo Reef may have an influence on coral bacterial shifts, particularly in June when algal detritus increases. For example, sequences related to heterotrophic *Vibrio* species represented up to 70% of clones derived from libraries in June, potentially indicative of a response to available nutrients released by algal die-off. Diversity indices calculated consistent values of associated bacteria between coral species and sampling times and revealed the highest diversity of microbial ribotypes in March and

October when the average daily temperature was highest and lowest, respectively throughout the sampling period. Similar to previous studies (Bourne et al. 2008; Koren and Rosenberg 2006) these results further indicate that temperature may directly or indirectly have an effect on the diversity of coral-associated bacterial assemblages.

A study carried out along the Northern Line Islands in the central Pacific Ocean, demonstrated that the ecology of microbial communities in seawater responds to regional oceanographic differences such as local upwelling and benthic community structure (Dinsdale et al. 2008). Similarly these factors may play a role in bacterial communities along Ningaloo Reef. With its central section being located only one kilometre off the continental shelf (Hearn and Parker 1988) the coastal current system along Ningaloo can generate transient upwelling in summer (D'Adamo and Simpson 2001; Woo et al. 2006). Environmental parameters have been shown to correlate with shifts in coral microbial associations and we suggest that changing bacterial communities in the water column driven by regional oceanographic characteristics may contribute to these shifts. Sample collection from *P. damicornis* in October displayed strong similarities in bacterial diversity and community structure between Rottnest Island and Ningaloo Reef, despite the great distance between the sites and their difference in ecosystem structure. All coral tissue samples collected in October grouped closely on the PCA plot. The Leeuwin current which meanders polewards along the Western Australian coast (see Figure 2.1) transports warm water along with marine larvae and picoplankton from more northern latitudes towards the south (Paterson et al. 2008); this could include bacterial communities in the water column. Assuming that coral-associated microbial bacteria are influenced by microbial communities present in the water column, the Leeuwin Current as part of the oceanographic regime of the Western Australian coast could partly explain the conserved coral-associated microbial consortia among coral species from Ningaloo Reef and geographically distant corals from Rottnest Island.

The present study provides the first overview on coral-associated microbial community structure and diversity of Western Australian corals. Our findings suggest seasonal changes to be involved in driving the microbial consortia in the three coral species *Pocillopora damicornis*, *Acropora tenuis* and *Favites abdita*, rather than coral species and spatial separation. Neither a distinct consortium of bacteria, nor coral bacterial species-specificity was detected in the coral species investigated; therefore, long term homeostasis between the coral hosts and their associated bacteria was not confirmed. Temporal shifts in coral-bacterial associations are potentially driven by the bacterial community present in the water column, which again may be influenced by oceanographic characteristics, benthic community structure and temperature. Currently it remains uncertain whether the coral host facilitates particular bacteria with a favourable habitat to allow for a mutual, beneficial relationship or whether it merely provides suitable living conditions for invading opportunistic microbial consortia which regulate the coral microbial associated community structure by competing for the same ecological niche. A better understanding of these coral-microbial relationships is important for the conservation, restoration and management of coral reefs worldwide.

CHAPTER 3

Coral-bacterial shifts through a coral mass spawning event

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Publication in review.

3.1 Abstract

Coral-associated bacterial communities of three coral species, the broadcast spawning coral *Acropora millepora*, the brooding coral *Pocillopora damicornis* and the ahermatypic coral *Tubastrea faulkneri*, were investigated before and after a mass spawning event on Ningaloo Reef in Western Australia. Two coral colonies of each species and an additional two samples for *P. damicornis* after planulation were collected to assess shifts in diversity and community structure of coral-associated bacteria with the aim to determine the drivers of these changes. Comparisons were also made between reproductive strategies (brooding vs. broadcast). A 470 bp region of the 16S rRNA gene including the variable regions 1-3 was selected for pyrosequencing with approximately 200,000 16S-tags recovered in total and an average of 20,000 tags per sample. At the taxonomical level of class, minimal shifts in community structure were observed. However, at the lower level of genus, distinct shifts in bacterial communities were detected. Bacteria affiliated with the *α-Proteobacteria* increased in relative abundance after coral spawning and particularly the clade *Roseobacter* was found to be prominent in all coral species, including *P. damicornis* after planulation, suggesting this bacterial group to be important and play specific roles in coral reproduction and early coral life stages. Coral mass spawning as well as coral reproduction were determined as drivers for changes in coral-associated bacterial communities.

3.2 Introduction

Corals exhibit a range of reproductive strategies, which include both sexual and asexual propagation. Brooding coral species show internal fertilization and expel well-developed larvae at various times of the year, usually over the summer months. Most corals however reproduce during annual spawning events, by broadcast spawning their gametes for external fertilisation (Harrison et al. 1984). Coral mass spawning is a well known phenomenon and occurs worldwide. According to the geographical location, coral species along with other invertebrates, participate in well timed synchronous spawning events that usually occur in summer, once a year over a few nights following the full moon (Harrison et al. 1984). Coral reproduction is critical to the persistence and resilience of coral reefs and is regulated by several life processes such as gamete production, fertilization, planktonic larval dispersal, larval settlement, post-settlement growth, and survival. Disruption in these early life stages can result in compromised or failed recruitment and profoundly affect the distribution and survival of corals.

Previous studies imply a stimulation of microbial processes within reef waters after episodic spawning events (Glud et al. 2008; Patten et al. 2008a). The input of large quantities of particulate organic matter in the form of degrading gametes enhance pelagic and benthic autotrophic and heterotrophic activities (Wild et al. 2008), and can result in rapid oxygen depletion in the water column (Simpson et al. 1993). After a coral mass spawning event on the Great Barrier Reef (GBR) bacterial abundances in reef water increased two-fold and remained elevated for three days, before declining to below pre-spawning values (Patten et al. 2008a).

Coral-associated microbes have been studied with regard to their role in coral health and disease (Rosenberg et al. 2007), coral mucus antimicrobial properties (Ritchie 2006) and their involvement in the biogeochemical cycling of nutrients (Olson et al. 2009; Raina et al. 2009).

Furthermore, microbes have been suggested to co-evolve with their coral host (Rosenberg et al. 2007) and to benefit the coral in adapting to environmental changes in the ecosystem (Reshef et al. 2006). Previous studies suggested that bacterial communities in corals are distinct from those inhabiting the surrounding seawater (Bourne and Munn 2005) and that some corals harbour specific bacteria species, despite temporal or geographical separation (Frias-Lopez et al. 2002; Rohwer et al. 2002). Other studies compared coral-associated bacteria between coral species in different locations and time points, showing that bacterial consortia varied with location (Littman et al. 2009) and time (Ceh et al. 2011), and therefore indicating that coral–microbial community structures may be a result of environmental drivers (Ceh et al. 2011; Hong et al. 2009) or species- and site-specific (Sunagawa et al. 2010). Understanding the acquisition, maintenance and change of microbial communities through development and life stages is fundamental to understanding the important functional roles that these partnerships have in overall coral health. Energy demanding physiological processes such as reproduction may affect the coral's metabolism which may impact other members of the coral holobiont (including *Symbiodinium*, Bacteria, Archaea, Fungi and viruses which form a functionally relevant mutualistic relationship with coral (Bourne and Munn 2005) known as the coral holobiont (Rohwer et al. 2002).

This study investigated whether coral-associated bacteria shift in diversity and community structure through a coral mass spawning event. More specifically we examined if related environmental factors such as microbial shifts in the water column rather than direct shifts related to coral reproduction were the drivers for coral-microbial changes. We assessed microbial communities in the broadcast spawning coral, *Acropora tenuis*, the brooding coral *Pocillopora damicornis* and the ahermatypic coral *Tubastrea faulkneri* before and after coral mass spawning. Additional samples were collected from *P. damicornis* after their respective reproductive activity. The broadcast spawning coral *A. tenuis* participates in the synchronous annual coral mass spawning, whereas the brooding coral *P. damicornis* repeatedly releases planula larvae through summer. In

comparison with broadcast spawners, *P. damicornis* corals release their planulae one week after the coral mass spawning event. *T. faulkneri* does not associate with the algal symbiotic partner *Symbiodinium* which has previously been suggested to be involved in structuring coral microbial communities (Banin et al. 2001; Raina et al. 2010). Therefore, relationships between this ahermatypic coral and associated microbes might be of a rather unspecific and opportunistic nature. Like *P. damicornis*, the ahermatypic coral *T. faulkneri* broods and releases planulae, however, the timing of reproduction is unknown for the Ningaloo Reef system and reproductive activity was not observed through one month of observation. Therefore, *T. faulkneri* served as a suitable model organism to investigate changes in microbial communities on corals, independently of reproductive strategy or activity.

Bacterial diversity was assessed by a 16S rRNA gene pyrosequencing approach allowing for large-scale exploration of taxonomic diversity, including rare and previously undiscovered taxa. This study is the first to investigate the dynamics of coral-microbial associates through coral reproductive stages and unravels the microbial involvement in the coral holobiont through a large scale ecological event.

3.3 Materials and Methods

3.3.1 Sample site and sample collection

All corals were pre-tagged and sampled on a reef flat (5-6 m water depth) near Coral Bay (23°07'S, 113°07'E), Ningaloo Reef, Western Australia. Samples were collected in replicates of two (one piece for each of two colonies) two days before and two days after the coral mass spawning event in March 2009. Three coral species, *P. damicornis* (brooder), *A. tenuis*, and *T. faulkneri* were

collected. Two additional *P. damicornis* colonies were removed from the reef structure and kept in an open plastic container (80 x 50 x 50 cm) on the reef flat during the day and assessed for reproductive activity on the beach at night time. The container was kept in knee deep water to maintain the ambient water temperature and returned to the reef at sunrise. *P. damicornis* were sampled on the reef two days after the last reproductive activity.

Two similar sized coral nubbins (approximately 2 cm in size) were removed from two coral colonies of each species using a bone clipper. Coral nubbins were instantly placed in individual, sterile zip-lock plastic bags under water and rinsed three times with artificial seawater (0.2 µm filtered and autoclaved) on the surface and placed on ice. The coral samples were air brushed with 2 mL of ASW to remove the coral tissue including the associated microorganisms from the coral skeleton and the tissue slurry aliquoted into cryovials. All samples were stored at -80°C until required for analysis. Samples were processed within one hour of sampling.

3.3.2 DNA extraction and PCR and sequencing preparation

Frozen tissue samples from all sampled corals were aseptically transferred to 1.5 mL Eppendorf tubes and total genomic DNA extracted performed using the MO BIO PowerPlant DNA Isolation Kit as per the manufacturer's instructions (MO BIO Laboratories, CA, USA). Extracted DNA was quantified using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech) and stored at – 20^{o}C until required.

A 470 bp region of the 16S rRNA gene (16S rDNA) including the variable regions 1-3 was selected for tag pyrosequencing using the bacterial forward primer 63F which included the primer A adaptor on the 5`end along with a unique 8 bp barcode (5`-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNCAGGCCTAACACATGCAAGTC

) and the bacterial reverse primer 533R with the primer B adaptor on the 5`end (5`-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTTACCGCGGCTGCTGGCAC). All amplifications were run under the following conditions: 1 x Qiagen PCR Buffer (Qiagen, Germany), 1 U of HotStarTaq DNA Polymerase (Qiagen), 200 µM of each deoxynucleotide triphosphate (dNTP), 25 pmoles of each primer and MilliQ water up to 50 µL. Equal volumes of DNA (20 ng total) from each sample were used to as template to generate PCR amplicons (tags). Thermocycling conditions for the amplification consisted of an initial 'enzyme activation' at 95^{o}C for 5 min, followed by 30 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min and 72^{o}C for 1 min, followed by a final extension step of 72^{o}C for 10 min to facilitate. A total of 5 PCRs were performed for each sample and the replicate PCRs pooled to generate ~1 µg of template DNA. PCR products were purified using the MO Bio PCR purification kit as per the manufacturer's instructions. Note: three of the *P. damicornis* and one of the *T. faulkneri* samples failed to amplify and were excluded from subsequent analysis. The amount of DNA in each sample was quantified using the Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA). All samples with their respective bar codes (12 samples in total) were pooled in equimolar amounts for 454 pyrosequencing on a Roche GS-FLX system at the Australian Genome Research Facility (AGRF) Brisbane, Australia.

3.3.3 Sequence analysis

Quality testing of the obtained sequence data was performed in two stages. First, all sequences that were potential primer dimers and shorter than 60 bp in length were removed from the dataset. Sequences were next sorted by barcodes into their respective samples and any sequences with mismatches in primer sequence removed before removing the barcode and primer sequences. For comparison of diversity between samples, the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al. 2010) was used. Sequences were quality checked using the following parameters: minimum quality score of 25, minimum length of 150 bp and maximum

length of 400 bp. Sequences were grouped into operational taxonomic units (OTUs) at two sequences identity thresholds; 90% for assignment to class affiliations and 97% for assignment to species affiliations. In QIIME, sequences were clustered using the uclust algorithm and represented sequences selected aligned using pyNAST and chimeric sequences removed again with ChimeraSlayer before being taxonomically identified using the RDP taxonomic classifier at 80% confidence (Wang et al. 2007). Alpha diversity statistics in QIIME were calculated after random sub-sampling to ensure sequencing effort did not affect diversity comparisons. Once the data set was rarefied, the following alpha-diversity metrics were generated; total observed species (OTUs), Shannon-diversity (H`) and Simpsons evenness (D). Beta-diversity of the bacterial communities was analysed using q weighted UNIfrac analysis with principal components generated from the UniFrac distances and plotted in two dimensions. The pyrosequencing dataset was deposited in the NCBI Sequence Read Archive (SRA) database with the accession number (pending).

3.3.4 Statistical testing of datasets

The output datasets were statistically compared to determine if diversity patterns of the samples were consistent at both the 90% and 97% sequence clustering levels. A Manhattan and Bray distance correlation of dominant OTUs identified from QIIME (at 90% and 97% sequence identity) was performed in the vegan package of R (Oksanen et al. 2007).

3.4. Results

Samples from three adult coral species *A. tenuis*, *P. damicornis* and *T. faulkneri*, collected before and after coral mass spawning and after planulation for *P. damicornis* provided a total of 208,066 subunit ribosomal RNA (16S rRNA) sequence tags (Table 3.1). One fragment from each coral colony was collected with two replicate colonies sampled representing 16 samples in total.

Unfortunately, difficulties in the DNA extraction resulted in a reduced number of twelve samples; four samples for *A. tenuis* (colony 1 and 2 before and after spawning), three samples for *P. damicornis* (colony 1 before and after spawning and after planulation) and three samples for *T. faulkneri* (colony 1 and 2 before spawning, colony 1 after spawning). Quality checked sequences with barcodes removed were grouped into Operational Taxonomical Units (OTUs) for sequences displaying > 97% sequence identity.

Diversity indices (*Shannon diversity (H) and Simpson evenness (D)*) calculated the highest diversity for the coral *A. tenuis* followed by *T. faulkneri* and the lowest diversity was detected for *P. damicornis*. After coral spawning, diversity increased in *A. tenuis* and *T. faulkneri* and decreased in *P. damicornis*. The eventual increase in diversity after planula release in *P. damicornis* and the increasing diversity levels after spawning in *A. tenuis* indicate that reproduction (here defined as the release of gametes and planula larvae) might be linked to increased bacterial diversity in these coral species.

Replicate sequence libraries from two coral colonies were pooled to provide a clear general overview over the ten most abundant bacteria classes (Fig. 3.1). Retrieved bacterial sequences from all coral species revealed highly conserved bacterial profiles at the taxonomic level of Class through the

Table 3.1: Sample information

Samples/Species	*A. tenuis (1)*	*A. tenuis (2)*	*P. damicornis*	*T. faulkneri (1)*	*T. faulkneri (2)*	*A. tenuis (1)*	*A. tenuis (2)*	*P. damicornis*	*P. damicornis**	*T. faulkneri (1)*
Sampling time	before coral mass spawning					after coral mass spawning				
Cleaned sequences	22018	22010	17484	23448	21642	19578	15193	21769	20839	24085
Barcode	CGAGAGA TAC	ATACGAC GTA	TCTACGT AGC	TACTCTC GTG	TAGAGAC GAG	CATAGTA GTG	TCACGTA CTA	CGTAGAC TAG	TACGAGT ATG	TGTACTA CTC
OTU (>97% ID)	4375	5435	1341	5068	3717	3423	2589	1595	2713	6191
Shannon diversity (H)	3.402	3.398	2.877	3.291	3.278	3.424	3.503	2.81	3.043	3.42
Simpsons evenness (D)	0.9451	0.9421	0.9051	0.9241	0.9308	0.9403	0.9508	0.895	0.9161	0.9472

* after planulation

event of coral mass spawning. All coral species were dominated by *α*- and *γ-Proteobacteria* (Fig. 3.1). For coral samples collected before spawning α-*Proteobacteria* represented 29.7%, 30.7% and 35.8%, for *A. tenuis*, *P. damicornis* and *T. faulkneri*, respectively; *γ-Proteobacteria* were similarly dominant and represented 39.6%, 28.5% and 46.9% of retrieved sequences.

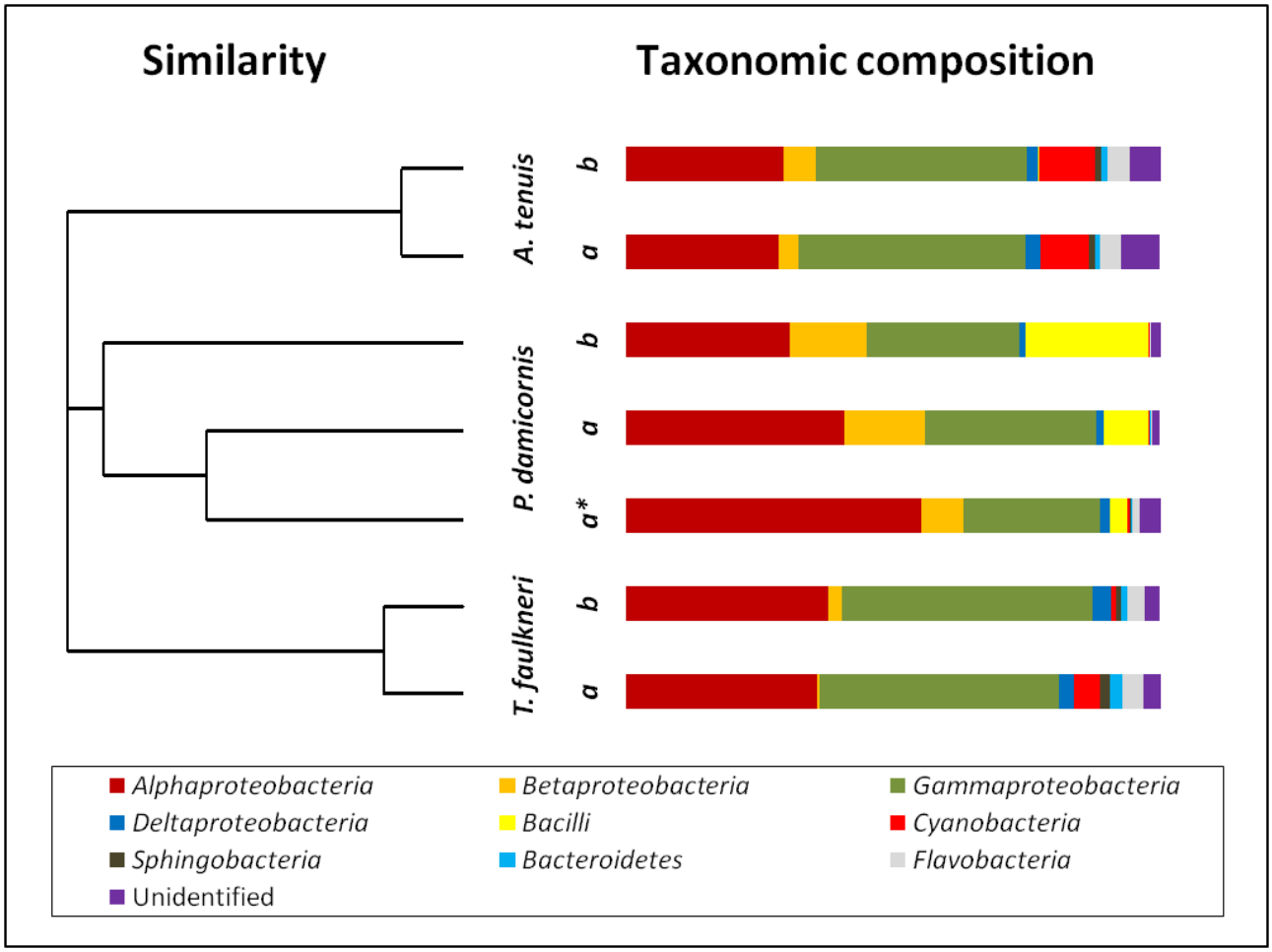


Fig. 3.1: Similarity in bacterial 16S rRNA gene sequences retrieved from three coral species before (b) and after (a) coral spawning and after planulation (a*). Replicate samples were pooled and dominant affiliations were grouped into OTUs >97% identity.

Sequence affiliations retrieved from *A. tenuis* grouped closely for both time points; however, replicates grouped closer before than after coral spawning. *P. damicornis* samples did not group for any two points in time (Fig. 3.2). *T. faulkneri* samples collected after coral spawning did not group with samples before coral spawning.

Similarities between corals before and after spawning were found to reflect highly conserved bacterial classes for coral species (Fig. 3.1) but also showed inconsistencies at the higher taxonomic genus level where changes in bacterial communities were displayed for all coral species (Fig. 3.2). As a result of these inconsistencies we analysed sequence affiliations of the most abundant (Fig. 3.3) and unique (Fig. 3.4) OTUs retrieved from corals sampled at different time points to further elucidate the identity of bacterial sequences responsible for changes in coral microbial assemblages.

Sequence tags grouped into OTUs at >97% identity were considered dominant when present at proportions of >1% in any sample collected and resulted in 39 most abundant OTUs out of a total number of 355 OTUs and covered 82% of all retrieved sequences. Sequences affiliations included the classes of *Acidobacteria*, *Flavobacteria*, *Cyanobacteria*, *α-, β- and γ-Proteobacteria* (Fig. 3.3). Eleven of 39 OTUs were present in all coral species and only six at all collection times (Fig. 3.3). *A. tenuis* samples consisted of 28, *P. damicornis* of 17 and *T. faulkneri* of 31 of the most abundant OTUs.

In the corals *A. tenuis* and *P. damicornis* the most abundant OTUs were generally present before and after spawning as well as after planulation in *P. damicornis.* However, in *T. faulkneri* high numbers of OTUs were not bound to sampling times. Sequences for *A. tenuis* were dominated by *Stenotrophomonas*, *Enterobacteriaceae, Roseobacter, Shewanellaceae and Rhodospirillales* and changes in proportions of the most abundant sequences occurred for 39.3% of OTUs. Sequences affiliated with the genera *Silicibacter* (increased by 1%) and *Pseudoalteromonas* (1%) only occurred after coral spawning.

Other sequences which increased in proportions after coral mass spawning included *Cyanobacteria* (1%), *Rhodomicrobium* (1%), *Roseobacter* (2%), *Rhodospirillales* (2%) and a group of unidentified *Bacteria* (2%). OTUs which decreased in proportion after coral mass spawning included *Flavobacteriaceae* (1%), *Delftia* (1%), *Enterobacteriaceae* (3%) and *Stenotrophomonas* (4%); with the later two occurring in large proportions before and after spawning (Table 3.3).

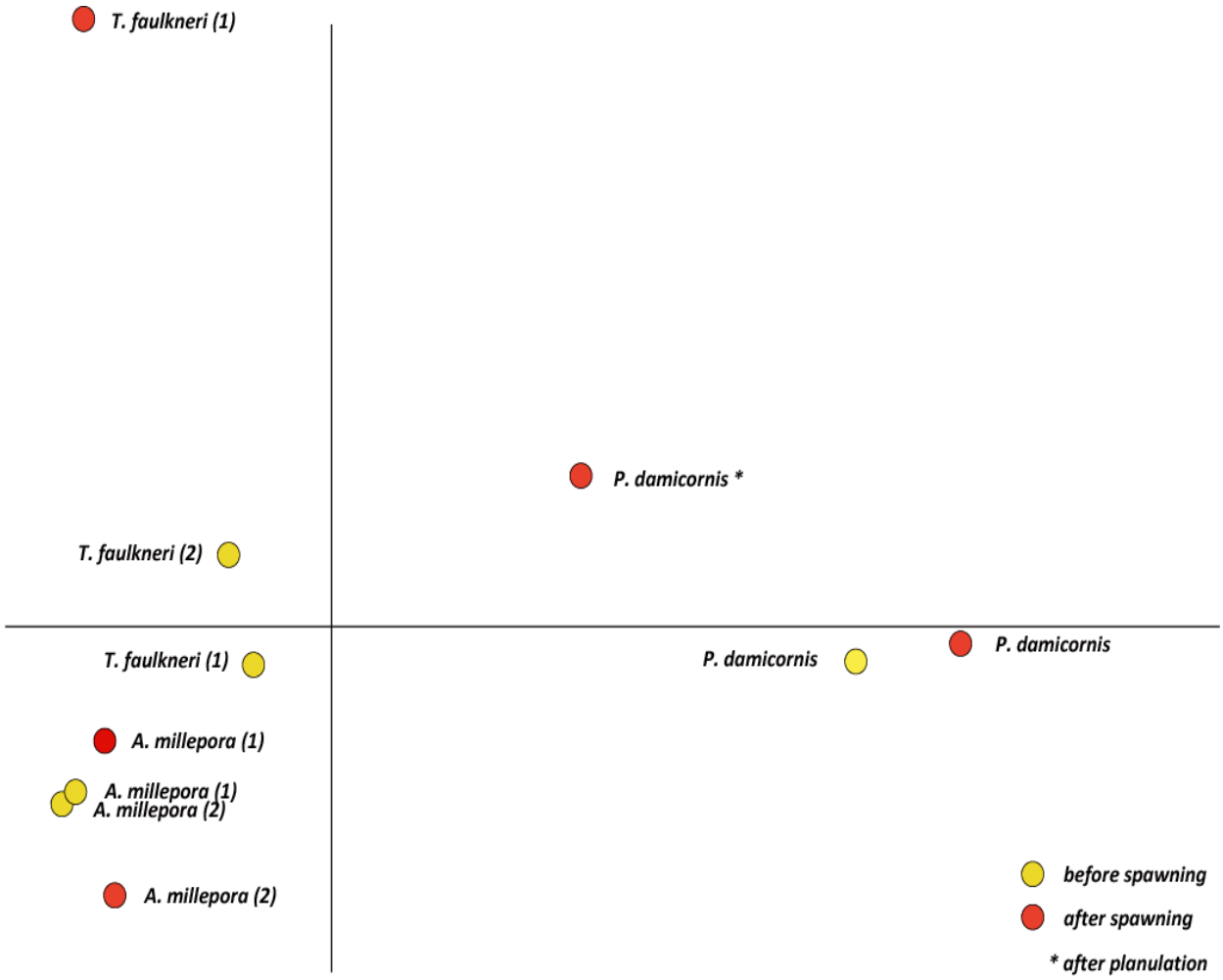


Fig. 3.2: PCA of 16S rRNA gene sequences, showing coral samples, before and after coral spawning and after planulation.

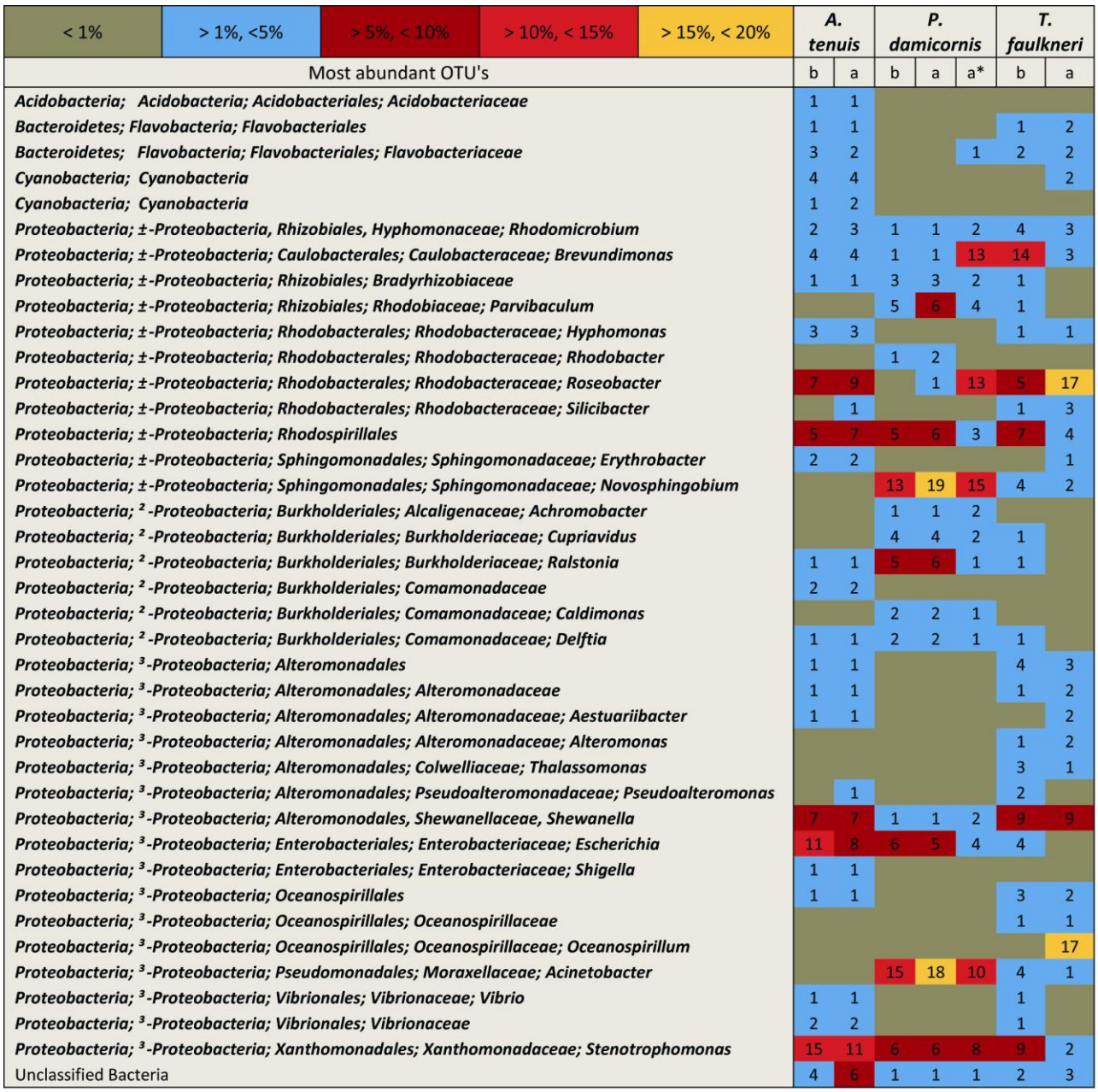


Fig. 3.3: Heat map showing the distribution of the most abundant bacteria OTUs acquired from three coral species before (b) and after (a) coral spawning and after planulation (a*). Sequences of proportions >1% of any sample where included in the map and color codes represent the percentage of sequence affiliations found.

Enterobacteriaceae were found to be most abundant in the coral *P. damicornis* and changes in proportions of the most abundant sequences occurred for 55.6% of OTUs.

Novosphingobium, *Acinetobacter*, *Stenotrophomonas*, *Brevundimonas*, *Parvibaculum*, and *Roseobacter* (1%) affiliated sequences only occurred after coral spawning. Other sequences that

increased in relative abundance after coral spawning were affiliated with *Parvibaculum* (1%), *Rhodobacter* (1%), *Rhodospirillales* (1%), *Novosphingobium* (6%), *Ralstonia* (1%) and *Acinetobacter* (3%). *Novosphingobium* and *Acinetobacter* were found in large proportions in all *P. damicornis* samples. The only OTU, decreasing in proportions after coral mass spawning represented the *Enterobacteriaceae* (1%).

Sequences affiliated with the group of *Flavobacteriaceae* (1%) only occurred after planulation. Two bacterial groups dramatically increased after planulation: *Brevundimonas* (12%) and *Roseobacter* (12%). Other sequences which increased after planulation included *Rhodomicrobium* (1%), *Achromobacte*r (1%), *Shewanella* (1%) and *Stenotrophomonas* (2%). OTUs decreasing in proportions after planulation consisted of the *Bradyrhizobiaceae* (1%), *Parvibaculum* (2%), *Novosphingobium* (4%), *Rhodobacter* (2%), *Rhodospirillales* (3%), *Cupriavidus* (2%), *Ralstonia* (5%), *Delftia* (1%), *Caldimonas* (1%), *Enterobacteriaceae* (1%), and *Acinetobacter* (8%). Sequences affiliated with the family *Roseobacter* and the genus *Brevundimonas* occurred in high proportions after planulation and bacteria affiliated with the order *Vibrionales* did not occur in *P. damicornis* samples at any time (Table 3.3).The most abundant OTUs in *T. faulkneri* contained bacteria affiliated with *Roseobacter*, *Shewanellaceae*, *Brevundimonas*, *Oceanospirillum*, *and Rhodospirillales* and changes in proportions of the most abundant sequences occurred for 91.3% of OTUs.

Sequences which were only present after coral spawning were affiliated with *Cyanobacteria* (1%), *Erythrobacter* (1%), *Aestuariibacter* (2%) and *Oceanospirillum* (17%).

Other OTUs which increased in proportions after coral spawning included *Flavobacteriales* (1%), *Roseobacter* (12%), *Silicibacter* (2%), *Rhodospirillales* (2%), *Alteromonadaceae* (1%), *Alteromonas* (1%), *Oceanospirillum***,** and unidentified *Bacteria* (1%). Sequences affiliated with the genus *Oceanospirillum* occurred only for the coral *T. faulkneri* after coral spawning and were found

to make up 17% of retrieved sequence tags. Sequences which disappeared after coral spawning were affiliated with the *Bradyrhizobiaceae* (1%), *Parvibaculum* (1%), *Cupriavidus* (1%), *Ralstonia* (1%), *Delftia* (1%), *Pseudoalteromonas* (2%), *Enterobacteriaceae* (4%), *Vibrio* (1%) and *Vibrionaceae* (1%). Other sequences which decreased in proportions after coral spawning were associated with *Rhodomicrobium* (1%), *Brevundimonas* (11%), *Novosphingobium* (2%), *Alteromonodales* (1%), *Thalassomonas* (2%), *Oceanospirillales* (1%), *Acinetobacter* (3%) and *Stenotrophomonas* (5%), (Table 3.3). The class *ß-Proteobacteria* did not occur in *T. faulkneri* after coral spawning.

A small proportion of all retrieved OTUs were unique to the coral species of origin; 12.1% for *A. tenuis*, 9.9% for *P. damicornis* and 11.5% for *T. faulkneri* (Table 3.2). To assess whether these unique OTUs were associated with specific sampling times in coral reproduction, the proportions of unique OTUs represented only before or after coral mass spawning, and unique OTUs that occurred at both times were analysed. In *P. damicornis* the time points after planulation and unique OTUs present at all sampling times were added. Proportions of unique OTUs decreased for *A. tenuis* and *T. faulkneri* after coral spawning but increased for *P. damicornis.*

Unique OTUs were found to be affiliated with fourteen bacteria classes (Fig. 3.4). The highest proportions were identified in *γ-Proteobacteria* (30), *α-Proteobacteria* (22) and *Flavobacteria* (22).

In *A. tenuis*, unique OTUs were more predominant before coral mass spawning with 21 OTUs representing 6 bacteria classes (Table 3.2). Nine unique OTUs affiliated with four classes were present after coral spawning and 13 OTUs affiliated with seven bacteria classes were found before and after spawning. *P. damicornis* displayed the highest number of unique OTUs after planulation (13), with seven classes represented. After spawning 10 unique OTUs affiliated with six classes were present, while before coral spawning 6 OTUs representing three classes.

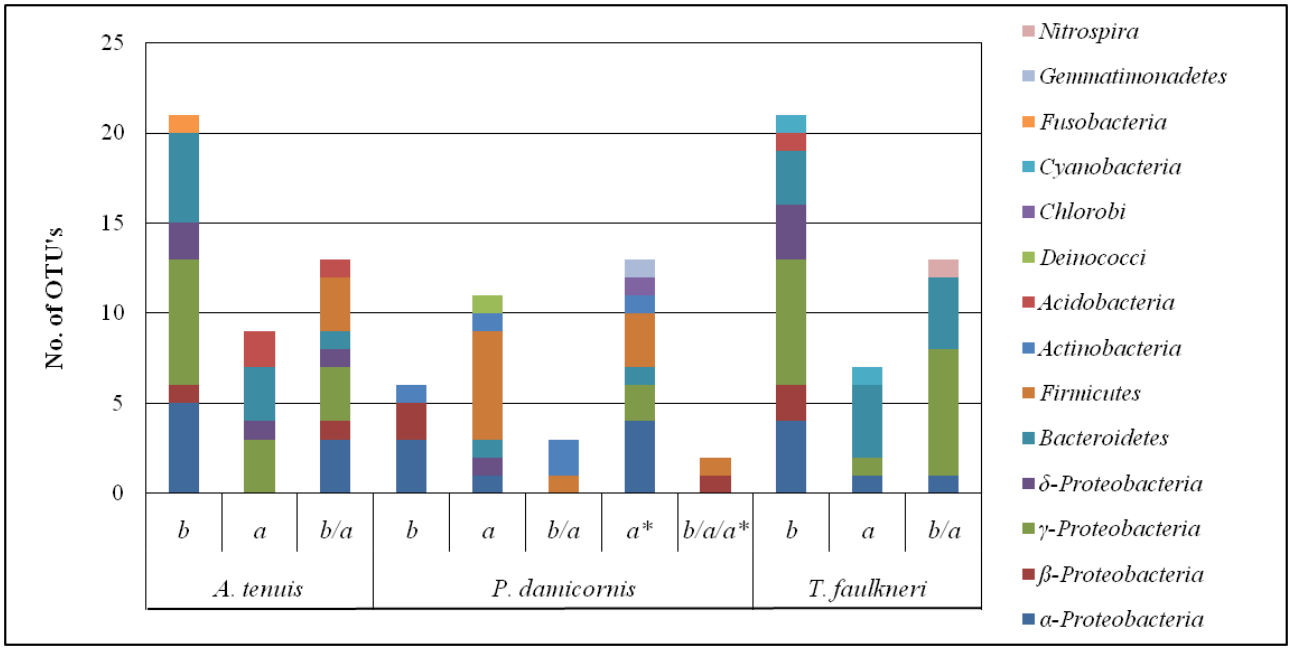


Fig. 3.4: Affiliations of sequences tags unique to one coral species. Proportions are displayed in total numbers of OTUs; (b= before spawning, a=after spawning, a*= after planulation).

Only two unique OTUS were found at all sampling times. Unique OTUs in *T. faulkneri* were highest before coral mass spawning (21) representing seven classes, followed by 13 for OTUs present at both times (four classes), before and after spawning and were lowest after coral spawning with 7 OTUs representing four classes.

Unique OTUs in *A. tenuis* were affiliated with *α-, ß-, γ-, δ- Proteobacteria*, *Flavobacteria*, *Acidobacteria* and *Fusobacteria*, which only occurred in *A. tenuis.* In *P. damicornis* unique OTUs were affiliated with *α-, ß-, γ-, δ- Proteobacteria*, *Flavobacteria*, *Firmicutes Actinobacteria*, *Deinococci*, *Chlorobi* and *Gemmatimonadetes;* the latter three OTUs only occurred in *P. damicornis* (Fig. 3.4). Unique OTUs in *T. faulkneri* were affiliated with *α-, ß-, γ-, δ- Proteobacteria*, *Flavobacteria*, *Acidobacteria*, *Cyanobacteria* and *Nitrospira*; the latter two OTUs only occurred in *T. faulkneri.*

Table 3.2: Number of unique OTU's at different sampling times in coral reproduction.

	Sampling Time(s)					
	b	a	b/a	a*	b/a/a*	Total no. of unique OTU's
A. tenuis	21	9	13	-	-	43
P. damicornis	6	11	3	13	2	35
T. faulkneri	21	7	13	-	-	41

Before spawing (b), after spawning (a), after planulation (a*)

Table 3.3: List of OTUs representing bacterial groups involved in coral reproduction.

		Coral species		
		A. tenuis	*P. damicornis*	*T. faulkneri*
Increasing OTU's after coral spawning				
Brevundimonas	±		12%*	
Novosphingobium	±		5%	
Rhodomicrobium	±	1%	1%*	
Rhodospirillales	±	2%		
Roseobacter	±	2%	1%, 12%*	12%
Acinetobacter	³		3%	
Oceanospirillum	³			17%
Stenotrophomonas	³		2%*	
Decreasing OTU's after coral spawning				
Brevundimonas	±			12%
Erythrobacter	±		3%*	
Novosphingobium	±		4%*	
Rhodospirillales	±		3%*	3%
Parvibaculum	±		2%*	
Rhodobacter	±		2%*	
Ralstonia	²		4%*	
Stenotrophomonas	³	4%		7%

***after planulation**

3.5 Discussion

Three coral species, *A. tenuis*, *P. damicornis* and *T. faulkneri* were examined to explore possible drivers for shifts in coral-associated bacterial assemblages through a coral mass spawning event. A previous study detected changes in coral-associated microbial communities through a Ningaloo Reef coral spawning event (unpubl. data), which raised questions about specific drivers causing these changes. Actual coral reproduction as well as coral mass spawning is a large scale ecological event which could potentially shift coral bacterial associates. Given that corals may acquire bacteria according to their specific requirements in different life stages, with reproduction accomplished colonies might rid themselves of bacteria associated with and important to reproduction and recruit alternative bacteria populations more suitable for the time after spawning. Coral bacteria might also shift due to corals releasing beneficial bacteria with their gametes (spawners) or planula larvae (brooders), and re-colonization with new bacteria. Alternatively, corals may simply return to pre-spawning bacterial populations as observed for temperature stressed and bleached corals (Bourne et al. 2008). However, coral microbial communities might not actively be structured by corals in need of particular partners, but rather through microbes colonizing corals as suitable habitats. In this scenario bacterial communities in the seawater, stimulated through the input of particulate organic matter from degrading gametes (Wild et al. 2008), might take advantage of corals potentially compromised and more susceptible to microbial colonization due to the stressful and costly event of reproduction.

No major shifts in coral bacterial communities were observed at the taxonomic level of class through the coral mass spawning event. This observation was consistent for all coral species which demonstrated similar microbial communities and any changes were only minor shifts in abundance of bacterial classes. Sequence affiliations at the lower taxonomic level of genus, incorporated into a PCA allowed for a higher resolution in coral-microbial associates and revealed changes through

coral spawning for all coral species, supporting the notion that large scale energy-demanding coral mass spawning event alters bacterial profiles in corals. Close grouping of replicate samples from *A. tenuis* and *T. faulkneri* before coral spawning indicated highly similar bacterial genera in similar proportions, likely structured by surrounding environmental factors, which is largely in accordance with previous observations ((Ceh et al. 2011) Chapter 2). *A. tenuis* replicates collected after coral spawning displayed a slightly different bacterial composition as replicate samples did not tend to group as closely as before spawning. Bacterial compositions in *T. faulkneri* changed more drastically and samples collected before and after coral spawning clearly separated from each other, showing less conserved and stable bacterial profiles than *A. tenuis* where bacteria seemed established in more consistent associations. Shifts in coral microbial assemblages in *P. damicornis* occurred after coral spawning and after planulation indicating that reproduction altered microbiota in this coral species (Table 3.3). Furthermore, *P. damicornis* displayed clear differences in bacterial composition compared to other coral species. This result corroborates the previous observation of distinctly differing bacterial profiles between the brooder *P. damicornis* and the broadcast spawning corals *A. tenuis* and *Favites abdita* in three of four time points over one year (Ceh et al. 2011), which were likely related to differences in life styles according to reproductive patterns. However, *P. damicornis* samples collected after planulation were similarly structured in bacterial communities to *A. tenuis* after spawning, strongly suggesting similar bacteria are involved in the process of reproduction between brooding and spawning corals.

Diversity indices showed higher diversity levels for the corals *A. tenuis* and *T. faulkneri* after coral spawning and after reproductive activity for *P. damicornis*, suggesting that coral reproduction might be related to higher diversity in coral-associated bacteria.

Only 28% of the most abundant OTUs (3% of all OTUs) were shared between all coral species investigated, and were associated with the *Flavobacteriaceae*, the *α-Proteobacteria*

Brevundimonas, Bradyrhizobiaceae, Roseobacter, and *Rhodospirillales,* the *β-Proteobacteria, Ralstonia* and *Delftia,* the *γ-Proteobacteria Shewanella, Enterobacteriaceae* and *Stenotrophomonas,* and one group of unclassified bacteria. Corals have previously been reported to harbour specific bacteria which differ from bacterial communities in the seawater (Bourne and Munn 2005) and these bacterial groups are likely to represent a consistent and significant component in coral-bacterial associations with *A. tenuis, P. damicornis* and *T. faulkneri,* whereas other bacterial groups which have not been found consistently dominant are likely to vary between coral species.

Investigating the taxonomical level of genus revealed a specific partition for bacterial classes associated with corals. For example within the family *Rhodobacteraceae* the genera *Hyphomonas* and *Silicibacter* were present in *A. tenuis* and in *T. faulkneri,* whereas *Rhodobacter* was only represented in *P. damicornis*. A similar pattern was observed for the family *Comamonadaceae* where three OTUs were specific to either *P. damicornis, A. tenuis,* or occurred in all corals. In the corals *A. tenuis and P. damicornis* 39% and 45% of the most abundant OTUs changed in proportion when comparing before and after spawning.

Interestingly, all bacteria types affiliated with the class *α-Proteobacteria* either increased (seven out of ten OTUs) or remained unchanged in relative proportion of retrieved sequences after spawning compared directly with pre-spawning samples. This indicates that *α-Proteobacteria* likely play important roles in coral reproduction with implications for the survival and increase of fitness in coral larvae; this is largely in accordance with previous work which reported the genus *Roseobacter* to be amongst the first acquired bacteria in early developing stages of the coral *Pocillopora meandrina* (Apprill et al. 2009).

The same pattern was observed for bacteria representing the *β-Proteobacteria.* However, bacterial groups affiliated with the *γ-Proteobacteria* either decreased or remained unchanged, with

the exception of the genera *Pseudoalteromonas* and *Acinetobacter* which increased in *A. tenuis* and *P. damicornis* respectively. Both genera are commonly known to associate with corals (Frias-Lopez et al. 2002; Rohwer et al. 2001; Rohwer et al. 2002) and other marine organisms. *Pseudoalteromonas* has previously been suggested to induce coral settlement (Webster et al. 2004) and to possess antimicrobial properties (Ritchie 2006; Shnit-Orland and Kushmaro 2009) and might be released by spawning corals to facilitate their offspring in the following settlement and survival processes.

In *T. faulkneri*, 91% of all the most abundant occurring OTUs changed in proportion through coral mass spawning, which again indicates unspecific and rather unstable relationships between this coral species and its bacterial associates. While affiliates belonging to the class *ß-Proteobacteria* did either not change or increased in proportions in *A. tenuis* and *P. damicornis*, the group disappeared in *T. faulkneri* after coral spawning.

Bacteria specifically related to reproduction in *A. tenuis* and *P. damicornis* were associated with *Rhodomicrobium* and *Roseobacter*; for example *Rhodomicrobium* increased in proportion in *A. tenuis* after spawning as well as in *P. damicornis* after planulation and *Roseobacter* increased for all coral species after coral spawning possibly reflecting high numbers of *Roseobacter* in the sea water. Furthermore, a large increase in proportion of *Roseobacter* after planulation potentially stresses the importance of this genus in *P. damicornis* reproduction, large numbers of *Roseobacter* occurred independently of the coral spawning event which was temporally separated from planulation in *P. damicornis* by one week. Other bacteria, important in *P. damicornis* reproduction, were affiliated with the genera *Brevundimonas* and *Novosphingobium* which were represented in much higher proportions after planulation. Increasing *Roseobacter*, no changes in *Shewanella*, and a decrease in *Enterobacteriaceae* proportions were consistently observed in all coral species through coral spawning. These bacteria seem to follow consistent and specific patterns through coral spawning for

all coral species and, along with other groups (listed in Table 3.3), might play primary roles in coral reproduction and the early establishment of the coral holobiont. Considering the challenging diversity of bacterial assemblages in adult corals this is particularly important, as the characterization of specific and relevant bacteria might be easier to achieve in early life stages with bacterial assemblages being less complex.

Unique OTUs occurred in all coral species at all sampling times and made up an average of 11% of all OTUs. Some unique OTUs were consistently present through sampling times and might represent stable and species-specific groups, however, others were likely related to coral spawning or planulation and occurred exclusively either before or after coral spawning, and after planulation in *P. damicornis*. Unique OTUs present at only one sampling time and in low proportions were most likely random transients. In the coral species *A. tenuis* and *T. faulkneri*, unique OTUs were highest in number before coral spawning and decreased after coral spawning. *A. tenuis* harboured distinct and diverse OTUs through coral spawning, whereas stable OTUs retrieved from *T. faulkneri* decreased in diversity. Unique bacterial groups are likely to be suppressed and outcompeted in the aftermath of a coral mass spawning event when available nutrients triggered by high inputs of organic matter allow rapid growth of certain bacterial species outcompeting others. Unique OTUs could also serve beneficial and specific functions, increasing the fitness in coral larval development and therefore be shed with the release of gametes and replaced in adult corals after spawning. *P. damicornis* displayed the lowest number of OTUs compared to other coral species and most unique OTUs were found after coral spawning and after planulation supporting both coral mass spawning and coral reproduction as drivers in coral microbial changes.

This is the first study to directly compare shifts in coral bacterial associations of coral before and after spawning. Coral spawning and reproduction represent energy demanding events, which

impact on the microbiota of corals. Coral species displayed similar classes of bacteria, though at the lower taxonomical level of genus, distinct differences in associated bacterial communities were observed. Abundant and unique OTUs likely represent bacteria which have some role in coral reproduction since they specifically appeared before or after coral spawning or in distinctly high numbers in between sampling times. The ahermatypic coral *T. faulkneri* was shown to be colonized by a diverse community of bacteria potentially representing unspecific and opportunistic associations, following bacterial profiles in *A. tenuis*, which supports the theory that corals might be colonized by bacteria, rather than corals actively structuring their microbial partners.

This 16S rRNA pyro-tag sequencing approach represents a powerful tool to elucidate distinct shifts in bacterial communities, and in this study provides an insight into how bacterial communities shift over a mass spawning event for three coral species on Ningaloo Reef. These results lay the groundwork for future research investigating potentially important functional roles of the identified bacterial groups, and their implication in coral reproduction and the early establishment of corals.

CHAPTER 4

Bacteria, released with coral spawn potentially increase the fitness of coral offspring

Publication in prep..

4.1 Abstract

Bacteria are diverse and abundant and play important functional roles in the multi-partite association of the coral holobiont. The specificity of coral-associated assemblages is still unclear though and little is known about the inheritance of specific bacteria from the parent colony to their offspring. This study investigated whether broadcast spawning and brooding corals release specific and possibly beneficial bacteria with their offspring to secure their maintenance across generations. The two coral species *Acropora tenuis* and *Pocillopora damicornis* were maintained in 0.2 µm filtered seawater during their release of gametes and planulae respectively. Water samples excluding gametes and planulae were subsequently collected and bacterial diversity assessed through a pyrosequencing approach amplifying a 470 bp region of the 16S rRNA gene including the variable regions 1-3. An average of 20.000 tags per sample was obtained and the affiliation of bacteria released by adult corals determined. Bacteria were isolated from *P. damicornis* planulae to identify which taxa had been uptaken from mother colonies. Compared to the high bacterial diversity harboured by corals, only a few taxa of bacteria were released by adult corals. Both *A. tenuis* and *P. damicornis* released similar bacteria and the genera *Alteromonas* and *Roseobacter* were abundant in large proportions in both species. Bacterial isolates retrieved from *P. damicornis* planulae were associated to the genera *Vibrio* and *Alteromonas* and showed up to 98% similarity to *Vibrio* and *Alteromonas* species released by mother colonies. This study suggests that adult corals may release bacteria with their offspring which potentially benefit the fitness in early coral life stages.

4.2 Introduction

Coral animals live in close and complex relationships with their associated microbial partners including dinoflagellates (zooxanthellae), Bacteria, Archaea, fungi, and viruses all together known as the dynamic assemblage of the coral holobiont (Knowlton and Rohwer 2003). These microorganisms inhabit microhabitats with the coral, such as the carbonate skeleton, the internal tissue, and the surface mucus layer (Rosenberg et al. 2007). Their beneficial contributions to the coral host, including nitrogen fixation (Lesser et al. 2004), defense (Ritchie 2006) and coral settlement (Webster et al. 2004) have been widely reported. Despite their benefits however, bacteria have also been implicated with coral disease and death.

Overall the mechanism controlling coral-bacterial interactions are still not clear and currently it is unknown whether true symbiosis or long-term species-specificity between corals and bacteria exists. The acquisition of symbionts between generations is usually referred to as either horizontal transmission where the host acquires the symbionts from the surrounding environment, or as vertical transmission with microbial partners being passed on to the next generation via the egg. The well studied association between the bobtail squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischerii* is regarded a model for the establishment, development and maintenance of horizontally transmitted symbiosis (McFall-Ngai and Ruby 1998); the establishment of this partnership has been shown to involve a step-wise elimination of potential interlopers, ensuring the exclusive symbiosis with *V. fischerii*, as opposed to thousands of other potential microbial partners (Nyholm and McFall-Ngai 2004).

Previous studies revealed that broadcast spawning corals do not transfer bacteria with their gametes (Apprill et al. 2009; Sharp et al. 2010) and bacteria are only acquired after settlement and metamorphosis into the juvenile polyp. Vertical transmission in brooding corals has not been

investigated to date, however, there have been reports in several marine invertebrates, such as bryozoans (Haygood and Davidson 1997), ascidians (Hirose 2000) and sponges (Sharp et al. 2007) of vertical transmission. Intergenerational maintenance of selected bacteria types might indicate important symbionts crucial to the fitness and survival of the coral host. Therefore, this study explored whether corals initiate associations with bacteria via inheritance from parent colonies by releasing beneficial bacteria with their gametes and planulae, a previously unexplored and potentially alternative strategy to vertical transmission. Furthermore, bacteria from *P. damicornis* planulae were isolated, identified and compared to sequences retrieved from adult corals in planulation water to determine whether bacteria released by adult corals were taken on by coral offspring.

4.3 Materials and Methods

4.3.1 Sample site and sample collection

All corals were sampled on a reef flat (5-6m water depth) near Coral Bay (23°07'S, 113°07'E), Ningaloo Reef, Western Australia. Two coral colonies from the coral species *Acropora tenuis* and *Pocillopora damicornis* colonies were removed from the reef structure and kept in open plastic containers (80 x 50 x 50 cm) on the reef flat during the day and assessed for reproductive activity on the beach at night time. The containers were kept in knee deep water to maintain the ambient water temperature and returned to the reef at sunrise. On the night of coral mass spawning, *A. tenuis* colonies were rinsed with and transferred into 0.2 µm filtered seawater and water samples of 1L collected from the container after coral spawning. The same procedure was conducted for planulating *P. damicornis* corals one week later. Water samples were filtered through Sterivex (0.22 µm) filter columns (Millipore) and all samples were stored at -80°C until required for analysis. Samples were processed within one hour.

4.3.2 Collection of P. damicornis planulae and bacteria isolation

Planula larvae were collected, transferred and maintained in a mixture of 1:1 planulation water and filtered seawater in glass dishes for 24 hrs, then washed in sterile seawater (x3) and crushed before spread plating on Marine Agar (Difco Marine Agar 2210). All seven morphologically dominant bacterial colonies were isolated to purity and identified by 16S rRNA gene sequencing as *Alteromonas sp.* and *Vibrio* affiliated bacteria (accession numbers pending).

4.3.3 DNA extraction, PCR and sequencing of water samples

DNA was extracted from seawater filters by the addition of 200 mL lysozyme (10 mg mL^{-1}), incubation at 37°C for 45 min, addition of 200 mL of proteinase K (0.2 mg mL^{-1}) and 1% SDS and incubation at 55°C for 1 h. Lysates were recovered into fresh Eppendorf tubes and DNA was extracted with a standard phenol:chloroform:isoamyl alcohol procedure and precipitated with 0.8 vol. of isopropanol.

A 470 bp region of the 16S rRNA gene (16S rDNA) including the variable regions 1-3 was selected for tag pyrosequencing using the bacterial forward primer 63F which included the primer A adaptor on the 5`end along with a unique 8 bp barcode (5`-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNCAGGCCTAACACATGCAAGTC) and the bacterial reverse primer 533R with the primer B adaptor on the 5`end (5`-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTTACCGCGGCTGCTGGCAC).

Sequence analysis and Statistical testing of datasets followed the procedures described in Chapter 3.

4.3.4 DNA extraction, PCR and identification of bacterial isolates

DNA was extracted from cultures grown overnight in marine broth (MB) at 28^0C using a Qiagen Soil Kit according to the manufacturer's instructions. The 16S rRNA gene of each isolate was sequenced using the 63f and 1387r oligonucleotides as sequencing primers. Cultures were stored in 30% v/v Glycerol at -80 ^{0}C. Obtained sequences were compared to sequences in the GenBank library (97% sequence identity) and to sequences retrieved from *P. damicornis* planulation water, using the GenBank alignment tool for the comparison of two or more sequences.

4.4 Results

Two water samples from spawning *Acropora tenuis* and planulating *Pocillopora damicornis* provided an average of 20,000 ribosomal RNA (16S rRNA) sequence tags. Sequences grouped at the 97% level of identity revealed thirteen OTUs from three classes (*Flavobacteria, α-Proteobacteria, γ-Proteobacteria*), occurring in proportions larger than 1% for either sample (Fig. 4.1). Water derived from *A. tenuis* displayed nine OTUs with large proportions in the *Flavobacteria* (61%) and *α-Proteobacteria* (28%). Within the *α-Proteobacteria*, sequences affiliated with *Roseobacter sp*. were dominant, representing 24% of all retrieved sequences. Other OTUs were affiliated with the *α-Proteobacteria Rhodomicrobium* and *Rhodospirillales*, and the *γ-Proteobacteria Shewanella* and *Alteromonas* and one group of unclassified bacteria. Water derived from *P. damicornis* was dominated by sequences affiliated with *α-Proteobacteria* from the *Roseobacter* clade (39%) and the *γ-Proteobacteria, Alteromonas* (32%), *Vibrio* (7%) and *Beneckea* (5%). Other OTUs for *P. damicornis* water samples were associated with the *Flavobacteriales*, the *α-Proteobacteria Rhodomicrobium, Rhodospirillales,* and *γ-Proteobacteria* affiliated with *Alteromonodales, Shewanella, Alteromonadaceae, Alteromonas, Vibrio, Beneckea* and a group of

unidentified *Bacteria*. Both coral species released into the water similar bacterial communities including *Flavobacteriaceae, Rhodomicrobium, Roseobacter, Rhodospirillales* and *Shewanella*, although these affiliated sequences were in different relative abundance.

< 1%	> 1%, <5%	> 5%, < 10%	> 10%, <25%	> 30%		
OTU's					***A. tenuis***	***P. damicornis***
Flavobacteria; Flavobacteriales					35	1
Flavobacteria; Flavobacteriales					20	
Flavobacteria; Flavobacteriales; Flavobacteriaceae					6	2
Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Rhodomicrobium					2	1
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseobacter					24	39
Alphaproteobacteria; Rhodospirillales					2	1
Gammaproteobacteria; Alteromonadales						1
Gammaproteobacteria; Alteromonadales; Alteromonadaceae						1
Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Alteromonas					1	32
Gammaproteobacteria; Alteromonadales; Shewanellaceae; Shewanella					3	1
Gammaproteobacteria; Vibrionales; Vibrionaceae, Beneckea						5
Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio						7
Unclassified Bacteria					2	1

Fig. 4.1: Heat map showing the distribution of the most abundant bacterial OTUs acquired from coral spawning/planulation water. Sequences of proportions >1% of any sample where included in the map and color codes represent the percentage of sequence affiliations found.

Interestingly, similar bacterial diversity was also observed for adult corals on the reef, two days after coral spawning and planulation (see Chapter 3). Particularly striking was the large abundance of *Roseobacter* in corals *A. tenuis* (24%) and *P. damicornis* (39%).

Bacterial isolates (n=7) cultured from *P. damicornis* planulae affiliated closely with *Alteromonas sp.* and *Vibrio sp.*, both members of the *γ-Proteobacteria* (Table 4.1). The five most abundant OTUs retrieved from the water of spawning *A. tenuis* and planulating *P. damicornis* demonstrated >97% sequence identity with the *Alteromonas* bacterial isolate and >96% identity with sequences within the genus *Vibrio*.

Table 4.1: *P. damicornis* planulae isolates.

Isolate No.	Closest match in GenBank	Level of ID	Level of ID*
1	*Vibrio alginolyticus*	99%	96%
2	*Vibrio sp. 1tc4*	99%	
3	*Vibrio harveyi*	99%	97%
4	*Vibrio sp. CF411*	100%	
5	*Alteromonas sp. N138*	99%	97%, 97%
6	*Alteromonas sp. MA336*	100%	98%, 97%, 97%
7	*Vibrio harveyi*	99%	97%

* compared to the 5 most abundant *Vibrio* and *Alteromonas* sequences retrieved from mother colonies (culture- independent analysis)

4.5 Discussion

This study suggests that corals, regardless of their reproductive strategy, may release beneficial bacteria with their offspring. A limited number of taxa and similar bacterial profiles between species indicate specific bacteria to be involved in coral reproduction and possibly early coral life stages. Several bacterial types released with gametes and planulae were also detected in coral tissue, two days after spawning and planulation, respectively and the consistent retrieval of these bacterial sequences through coral reproduction and their presence of some in *P. damicornis* planulae suggest specific functions through coral reproduction and early coral life history.

Inter-species differences were mainly based on large proportions of *Flavobacteriales* affiliates in *A. tenuis*, possibly indicating spawner-, or species-specific roles of these bacteria in *A. tenuis* reproduction. Dominant bacteria released by *P. damicornis* included the genera *Roseobacter* and *Alteromonas*. *Roseobacter* clade affiliated bacteria species are abundant and diverse in seawater and various metabolic functions have been reported for this taxon (Piekarski et al. 2009), including antibiotic properties against coral pathogens (Nissimov et al. 2009). As highlighted in Chapter 3, *Roseobacter* species, increased in abundance in all coral species after coral spawning, possibly

providing antimicrobial activity against potentially pathogenic bacteria for coral compromised after energy demanding life stages such as spawning. These previously reported beneficial aspects to adult corals by Alteromonas- and Roseobacter associated clade affiliated species might be equally important in the vulnerable early life stages of larvae, polyps and juvenile corals. Broadcast spawning larvae travel long distances and if beneficial bacteria are to be successfully transferred to the next generation via their release with gametes, they have to be highly abundant and widely dispersed in seawater. This is easily assured, assuming that all coral species, participating in coral mass spawning release *Roseobacter* affiliated bacteria. Bacterial abundance after a coral mass spawning event on the Great Barrier Reef increased two fold and returned to pre-spawning levels after three days (Patten et al. 2008b). The brooder *P. damicornis* releases planulae one week after the coral mass spawning event, when *Roseobacter* abundance has likely dropped to normal levels. To still ensure the transfer of bacteria to its offspring, adult colonies of brooding corals, which occur in much lower numbers than spawners, would have to release larger proportions of bacteria. Brooding corals might take advantage of increased *Roseobacter* abundance in the seawater after coral mass spawning and acquire, and maybe even 'culture' these bacteria internally for the release with their own offspring. This could also explain the large increase of *Roseobacter* species (12%) in *P. damicornis* after coral mass spawning (Chapter 3). Planulae from brooding corals usually settle within hours of their release and in close proximity to mother colonies; if the *Roseobacter* clade is an essential element in the settlement and survival success of coral larvae, the dispersal distance of released *Roseobacter* might limit the settlement distance in *P. damicornis* planulae from mother colonies. Our data indeed shows that *P. damicornis* do release much higher proportions in *Roseobacter* species than *A. tenuis*. A similar pattern was observed in bacteria affiliated with the genus *Alteromonas*.

Previous studies reported bacteria associated with the genus *Alteromonas* to induce coral settlement (Webster et al. 2004). One isolate from *P. damicornis* planulae induced settlement and

metamorphosis in acroporid corals (*pers. com.* Hokoshiki) and was furthermore confirmed to be released by adult *P. damicornis* during planulation, again confirming the transfer of beneficial *Alteromonas* bacteria from adult *P. damicornis* to the next generation. This transfer could be of a vertical nature; nonetheless, *Roseobacter* species affiliated species were found to be the first colonizers in *Pocillopora meandrina* polyps even though not vertically transferred via eggs or sperm, showing that transfer of potentially beneficial bacteria to the next generation is not dependent on vertical transmission if the abundance of the bacterium is secured in the water column. This further supports the notion that adult corals may increase the fitness of their offspring by releasing beneficial bacteria during spawning and planulation. The beneficial role of transferred *Alteromonas sp.* and *V. alginolyticus* from adult colonies to planulae in *P. damicornis* is demonstrated and discussed in Chapter 5.

Roseobacter affiliated bacteria occur in large proportions for both corals which, together with findings in Chapter 3 demonstrate specific and important roles in coral reproduction, larval settlement and survival success. Clone libraries retrieved from *A. tenuis* and *P. damicornis* through one year (Chapter 1) showed the presence of *Roseobacter* clade affiliated species in three out of four sampling times confirming their proposed importance in several coral life stages.

In conclusion: Independently of reproductive strategy, parent corals likely transfer specific groups of bacteria to the next generation which are released during spawning and taken up by the offspring. This strategy is less specific than intergenerational vertical transfer, however, not as non-specific as random horizontal acquisition of seawater communities. The selective sorting of bacteria is likely to reflect the selection of potentially important bacteria which have an implication for increasing the fitness of coral offspring. The genera *Alteromonas* and *Roseobacter* might be the most relevant taxa in coral reproduction and early coral development.

CHAPTER 5

Living in a nutrient poor world: *Symbiodinium* symbionts in coral larvae take up nitrogen from bacteria

Publication in prep..

5.1 Abstract

Corals and microbes live in close and complex, but poorly understood relationships. Microbial symbionts are likely to increase the animals' fitness; however, the initial establishment of coral-microbial partnerships in early and vulnerable life stages of corals has received little scrutiny to date. Coral larvae of the common species *Pocillopora damicornis* and *Acropora millepora*, representing a brooding and a spawning coral, respectively, were incubated with isotope (^{15}N) pre-labelled *Alteromonas sp.*, or *Vibrio alginolyticus* bacteria (both previously isolated from coral larvae) to visualize potential early interactions between bacteria and corals. The onset of early coral-bacterial associations was visualized by advanced nano-scale secondary ion mass spectrometry (NanoSIMS) and the uptake and translocation of nitrogen via *Vibrio*- and *Alteromonas* bacteria into *P. damicornis* coral larvae and specifically into the coral-symbiotic *Symbiodinium* was detected. A significant increase in enriched nitrogen was observed in *P. damicornis* larvae within eight hours after incubation for both bacteria whereas *A. millepora* was only found to harbour enriched nitrogen from *V. alginolyticus* after 24 hours. These findings reveal a crucial role of coral-associated bacteria in providing nitrogen, an essential but limited nutrient in coral reef ecosystems, to the coral animal.

5.1 Introduction

Coral reefs are currently subjected to increasing anthropogenic pressure resulting in alarming rates of decline (Baker et al. 2008; Carpenter et al. 2008). Increases in atmospheric concentrations of carbon dioxide contribute to environmental changes such as ocean warming and acidification (Doney et al. 2009; Feely et al. 2009; Harvell et al. 2007); these disrupt the delicate symbiosis between corals and their microbial partners (De'ath et al. 2009; Hoegh-Guldberg et al. 2007; Kleypas and Yates 2009). Corals host unicellular algal symbionts (*Symbiodinium)*, Bacteria, fungi, Archaea and viruses, which all form a collaborative consortium known as the coral holobiont (Rohwer et al. 2002; Rosenberg et al. 2007). While *Symbiodinium* have long been known as beneficial obligate coral symbionts (Muscatine 1973), the roles of coral-associated bacteria are not well understood, even though it has been proposed that they fulfil important functions in maintaining coral health and resilience (Bourne and Munn 2005; Rohwer et al. 2002; Rosenberg et al. 2007).

There is increasing evidence that microbes perform important roles in biogeochemical cycles within the coral host (Raina et al. 2009). For example, some coral-associated bacteria and Archaea fix nitrogen (Lesser et al. 2004; Olson et al. 2009) that can be scavenged by the host. Nitrogen is essential for growth and development in all organisms; however, the ability to fix atmospheric nitrogen is strictly limited to some bacteria that express the nitrogenase enzyme complex. Some eukaryotes obtain nitrogen through their symbiotic interactions with nitrogen-fixing bacteria (Kneip et al. 2007). The tight nutrient cycling between symbiotic partners is likely to enable the coral holobiont to flourish in the oligotrophic waters surrounding coral reefs.

Bacteria associated with planktonic coral larvae are likely to increase the animals' fitness through assimilation and transfer of essential nutrients, thus conveying additional resources critical

for coral distribution and enabling persistence and biodiversity of wider tropical marine communities. Despite the profound global importance of these symbiotic relationships, information on the onset and establishment of coral-microbial symbiosis is limited. One recent study revealed the establishment of coral-associated bacterial partnerships in *Pocillopora meandrina* in late developmental stages of planulae (after 76 hrs) and suggested a functional role of bacteria in processes specific to this life stage, such as coral settlement (Apprill et al. 2009). Another study observed bacterial communities in broadcast spawning corals only after settlement and metamorphosis of the host (Sharp et al. 2010). Up until now the establishment of coral-bacterial partnerships in brooding corals has not been investigated.

Two coral species were used in this study: The brooding coral *Pocillopora damicornis,* which releases fully functional planulae (free-swimming larvae) containing *Symbiodinium* and *Acropora millepora,* a broadcast-spawning coral with external fertilization and larval development. *A. millepora* larvae acquire their algal symbionts from their surrounding environment and planulae of this species had not yet assimilated *Symbiodinium* at the time of our experiment. State of the art NanoSIMS technology, a microprobe for isotopic and trace element analysis at high spatial resolution, was applied to observe potential interactions between coral larvae and bacteria and to determine the functions of these, if any. Previous studies have explored Nano SIMS technology as a useful tool to elucidate symbiotic relationships between organisms (Foster et al. 2011; Lechene et al. 2007).

5. 3 Materials and Methods

5.3.1 Overview

Two bacteria strains isolated from coral larvae and identified as *Alteromonas sp*. and *Vibrio alginolyticus* (see below), were grown in media containing ^{15}N as the only nitrogen source. Planulae of *P. damicornis* and *A. millepora* were then incubated with isotope labelled *Alteromonas sp*., or *Vibrio alginolyticus* (1 x 10^6 bacteria mL^{-1} Filtered Sea Water) and maintained at 27°C in a 12:12 light:dark diurnal pattern. Samples were collected after 8 and 24 hours. Nitrogen incorporation in bacteria, transfer of ^{15}N into coral planulae and the distribution and final destination of nitrogen in coral tissue was determined using a combination of nano resolution second ion mass spectrometry (NanoSIMS) and fluorescence *in situ* hybridization (FISH) In addition we used transmission electron microscopy (TEM) to visualize morphological details in planulae at ultra-structural resolutions.

5.3.2 Bacteria isolation & identification

Pocillopora damicornis colonies were collected in March 2009, from Coral Bay, Ningaloo Reef (231070S, 1131070E) and kept in aquaria for five days until planulae were released. Planula larvae were collected, washed in sterile seawater (x3) and crushed before spread plating on Marine Agar (Difco Marine Agar 2210). Two morphologically dominant bacterial colonies were isolated to purity and identified by 16S rRNA gene sequencing as *Alteromonas* sp. (accession number pending) and by multilocus sequence typing as *Vibrio alginolyticus* (accession number pending). DNA was extracted from cultures grown overnight in marine broth (MB) at 28 ^0C using a Qiagen Soil Kit according to the manufacturer's instructions. The 16S rRNA gene of each isolate was sequenced using the 63f and 1387r oligonucleotides as sequencing primers (Marchesi et al. 1998). The three protein coding loci *rpoA* ((RNA polymerase a-subunit), pyrH (uridylate kinase) and recA were used

for multilocus sequence identification, amplified by PCR and sequenced. Cultures were stored in 30% v/v Glycerol at -80 °C. Obtained sequences were compared to sequences in the GenBank library (97% ID).

5.3.3 Bacteria labelling with stable isotope ^{15}N

Isolates were grown in either Celtone Base Powder (^{15}N, 98%+) or Celtone Base Powder unlabelled (Cambridge Isotope Laboratories), in artificial seawater (ASW) for 24 hours at 28°C, washed 3x in ASW and either fixed to confirm the uptake and incorporation of enriched stable ^{15}N isotope (Fig. 5.1) or used in coral experiments.

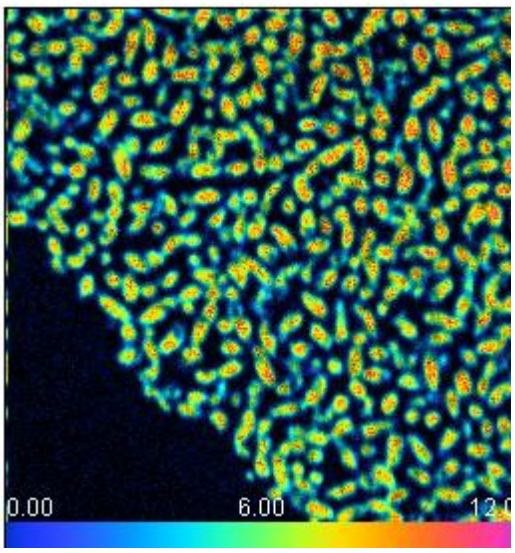


Fig. 5.1: NanoSIMS analysis: Incorporated ^{15}N in *Alteromonas sp*. bacteria. Hue saturation and intensity scale (HSI) of the ratio $^{15}N/^{14}N$, blue represents the natural isotopic abundance of nitrogen (0.0037) and enrichment of ^{15}N is represented by the shift in colour towards magenta (0.05). The image is 20x20µm and 256x256 pixels; acquisition time was 20 min.

5.3.4 Experiments

Coral colonies were collected from Orpheus Island (Great Barrier Reef, Australia) and transferred to the Australian Institute of Marine Science outdoor aquarium facilities. Aposymbiotic *Acropora millepora* coral larvae were raised for seven days in 1 µm filtered seawater (FSW) in 500

L tanks. *P. damicornis* larvae were collected just after their release and planulae of both species collected, washed three times in 0.2 µm FSW and transferred to 0.2 µm FSW in sterile 6-well titre-plates (BD Falcon). Coral planula larvae were kept at 27°C water temperature and 12:12 light:dark diurnal pattern. After 12 hrs bacteria were added to wells of the microtitre plate at a density of 1 x 10^{-6} cell mL^{-1} FSW. Twenty planula larvae per well were co-incubated with isotope labelled *Alteromonas* sp., or *Vibrio alginolyticus* (1 x10^{6} bacteria mL^{-1} FSW) for 8 and 24 hrs respectively. Incubations with unlabelled bacteria and planulae not exposed to any bacteria served as controls. Bacterial viability of inoculated cells was confirmed through re-isolation, quantification (dilution plating) and identification (100% 16S rRNA gene similarity).

5.3.5 Sample preparation

Bacteria and ten coral planulae for each treatment were sampled and fixed for either nano-scale second ion mass spectrometry (NanoSIMS) or fluorescent *in situ* hybridization (FISH), (10 mL 25% Glutaraldehyde + 10 mL 10% Paraformaldehyde + 180 mL ASW). All coral planula larvae were rinsed in PBS, dehydrated in a graded series of ethanol (50%, 70%, 90%, 100%, dry 100%) and dry acetone, infiltrated and embedded in resin (26g Araldite, 24g DDSA, 1.4g BDMA, 2 acetone:1 resin, 1 acetone:2 resin, pure resin) and cured for 36 hours at 60°C. All sections were cut from resin blocks with glass knifes on a Leica EM UC6 (Leica Microsystems, Wetzlar, Germany).

5.3.6 TEM analysis

Ultra-thin sections (70nm) were collected on Formvar coated copper grids (1x2 mm slotted) and analysed on a Jeol 2100 TEM (Tokyo, Japan).

5.3.7 NanoSIMS analysis

NanoSIMS analyses were performed at the Centre for Microscopy, Characterisation and Analysis (CMCA) at the University of Western Australia (UWA), using a CAMECA NanoSIMS 50 ion microprobe (CAMECA, Paris, France).The abundance of nitrogen in individual bacteria cells (Fig. 5.1) and in coral larval tissue was measured and mapped; the incorporation of ^{15}N tracers was measured by comparing two simultaneously acquired images mapping ^{15}N and ^{14}N, and determining the increase of $^{15}N/^{14}N$ ratio compared to the natural isotopic abundance. The enrichment of ^{15}N isotope is expressed as colour on a Hue, Saturation and Intensity (HSI) scale, where blue represents the natural isotopic abundance of Nitrogen (0.0037) and enrichment of ^{15}N is represented by the shift in colour towards magenta.

5.3.7.1 Preparation of NanoSIMS samples:

Bacteria and coral larvae were embedded in resin and sections of 600 nm cut and mounted on silicone discs and gold coated for NanoSIMS analysis. The incorporation of ^{15}N into individual bacteria cells and coral planula tissue was localized and measured using the Cameca NanoSIMS 50 ion microprobe. All samples were compared to a yeast standard with a known natural abundance ratio between $^{15}N/^{14}N$.

5.3.7.2 Analysis of NanoSIMS images:

Images were processed and analysed using the free software ImageJ including open *MIMS plugin* for *ImageJ* (*www.nrims.hms.harvard.edu/NRIMS_ImageJ.php)* which have been developed by Lechene and colleagues. We manually drew 96 regions of interest (ROI) on individual HSI images, three images per treatment were taken.

5.3.8 FISH analysis

FISH was performed on 600nm dehydrated coral larval sections on agarose-coated microscopy slides with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulphate) containing 35% formamide. Samples were probed with three different probe mixes, according to their previous treatments: *Alteromonas sp*. treated coral larval samples were probed with equimolar amounts of general bacterial probe mix EUB338I-III (5'- GCT GCC TCC CGT AGG AGT -3', 5'- GCA GCC ACC CGT AGG TGT -3', 5'- GCT GCC ACC CGT AGG TGT -3') CY3 double-labelled oligonucleotides, *Gammaproteobacteria* specific probe GAM42a (5'- GCC TTC CCA CAT CGT TT -3') CY5 double-labelled oligonucleotides, *Alteromonas* specific probe Alter2 (5'- CCA CAC TTT CGC ACA TGA GC -3') and the competitor probe to GAM42a, BET42a (5'- GCC TTC CCA CTT CGT TT -3') as unlabelled oligonucleotide. Coral larvae previously treated with *Vibrio alginolyticus* were probed with equimolar amounts of general bacterial probe mix EUB338I-III (5'- GCT GCC TCC CGT AGG AGT -3', 5'- GCA GCC ACC CGT AGG TGT -3', 5'- GCT GCC ACC CGT AGG TGT -3') FLUOS double-labelled oligonucleotides, *Gammaproteobacteria* specific probe GAM42a (5'- GCC TTC CCA CAT CGT TT -3') CY5 double-labelled oligonucleotides, *Vibrio* specific probe Vib572a (5'- ACC ACC TGC ATG CGC TTT -3') and the competitor probe BET42a (5'- GCC TTC CCA CTT CGT TT -3') as unlabelled oligonucleotide. Negative control samples were probed with NONEUB (5'- ACT CCT ACG GGA GGC AGC -3') CY3, CY5 and FLUOS double-labelled oligonucleotides or a mix of a nonsense probe, double-labelled with CY3 together with equimolar amounts of general bacterial probe mix EUB338I-III (5'- GCT GCC TCC CGT AGG AGT -3', 5'- GCA GCC ACC CGT AGG TGT -3', 5'- GCT GCC ACC CGT AGG TGT -3') Cy5 double-labelled oligonucleotides. The probes were added to the buffer at a final concentration of 5 pmol/µL^{-1} for Cy3 and Cy5, and 8.3pmol/µL for Fluos; the samples were hybridized over night at 46°C. The hybridization buffer was removed and the samples

incubated in wash buffer (0.7 M NaCl, 20 mM Tris-HCl, 50 mM EDTA, 0.01% sodium dodecyl sulphate) at 48°C for 20 minutes before they were dipped into ice cold MilliQ water, air-dried and stored at -20°C. For further application microscope slides were warmed to room temperature before adding antifadent Citiflour, (Citifluor Ltd, UK) and covered with a cover slip. The visualization of the samples was performed on a ZEISS LSM Meta Confocal Microscope (Carl Zeiss, Jena, Germany) and analysed with the LSM Image Browser software.

5.3.9 Statistical data analysis

All statistical analyses were performed using the free statistical software PAST (Ryan et al. 1995). Statistics information is found in the supplementary information (S.5.1).

5.4 Results

Evaluating and comparing the ^{15}N distribution in tissue types of *P. damicornis* planulae, we found significant increases in enriched nitrogen in *Symbiodinium* and in the outer ectodermis (Fig. 5.2, ^{15}N enrichment in magenta). The ^{15}N distribution in the enriched outer epidermis of *P. damicornis* was not uniform and was strongly correlated with the presence of elongated cellular structures that were identified as mucus-producing cells and nematocysts (Fig. 5.2; Fig. 5.3).

Ec
En
M
S
15N
0.005
0.0045
0.004
0.0035
0.003
Ecto I
Ecto O
Endo I
Endo O
Meso
Symb
*
*

Fig. 5.2: (previous page) Section of *P. damicornis* planula after 8 hrs exposure to ^{15}N labelled *Alteromonas sp*.. NanoSIMS analysis: Mosaic of HSI's of the ratio $^{14}N/^{15}N$, green represents the natural isotopic abundance of nitrogen (0.0037) and enrichment of ^{15}N is represented by the shift in colour towards magenta; each tile is 80x80 µm; 256x256 pixels; acquisition time was 30 min, scale bar 50µm. Ec=Ectodermis, En=Endodermis, M=Mesogloea, S=*Symbiodinium*.
Small image: Graph showing the $^{14}N/^{15}N$ isotope ratio within different tissue types in *P. damicornis* planulae after 24 hrs exposure to ^{15}N labelled *Alteromonas sp;* the highest increase in the $^{14}N/^{15}N$ ratio over the natural value occurs in *Symbiodinium* and nematocysts. Asterisks indicate significant differences between tissues; statistical information is found in the supplementary section) the line indicates the natural abundance of ^{15}N. Ecto=Ectoderm, Endo=Endoderm, Meso=Mesogloea, Symb=*Symbiodinium*, I=Inner/ O=Outer.

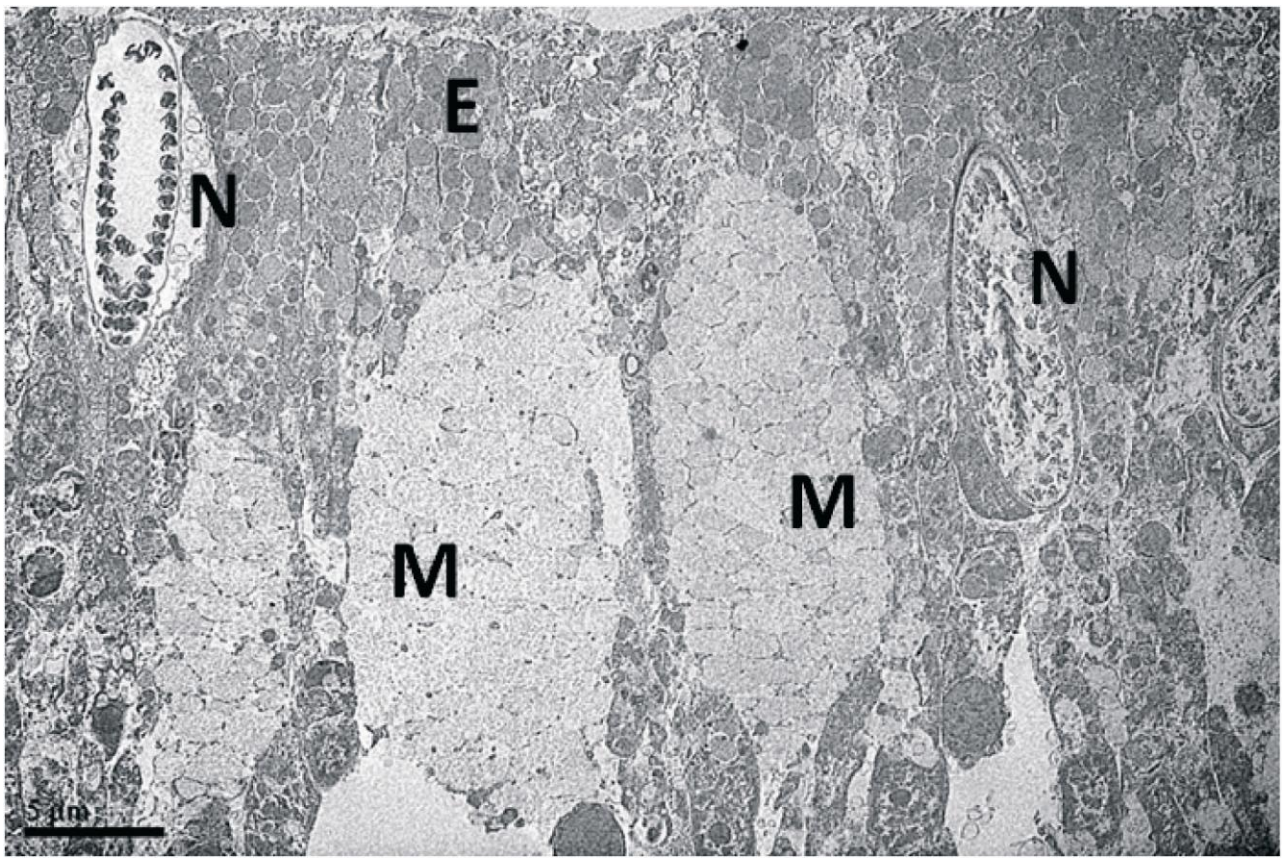


Fig. 5.3: Transmission electron micrograph: Nematocysts (N) and mucocytes (M) in the ectodermis (E) of *P. damicornis* planulae (70nm section); scale bar 5µm.

Within eight hours, $^{15}N/^{14}N$ ratios within *Symbiodinium* were elevated by up to a factor of 12 (compared to natural abundance); with an average four-fold increase for *Alteromonas* sp. and an average three-fold increase for *V. alginolyticus* treated planulae (Fig. 5.4 A1-3, V1-3).

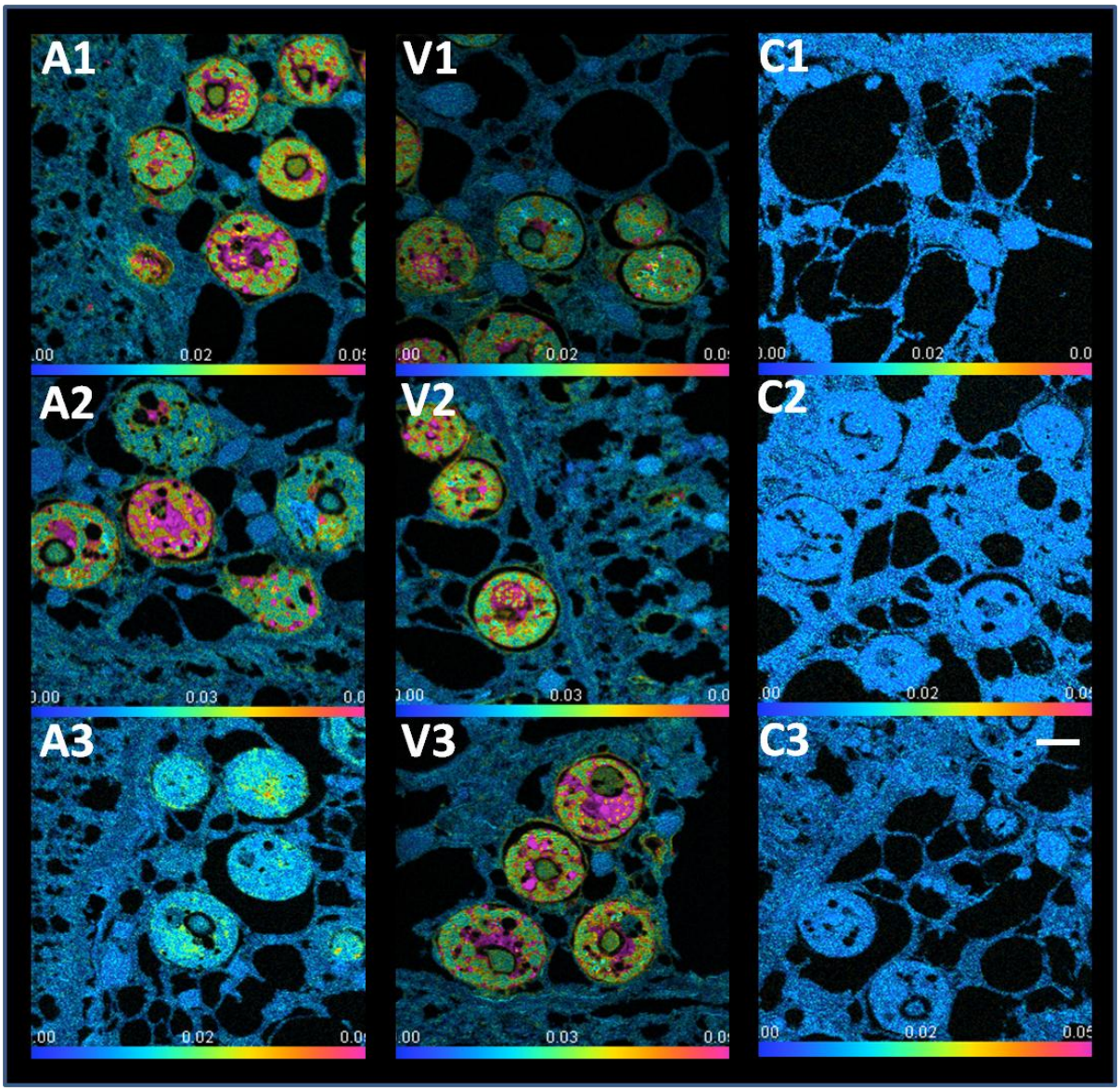


Fig. 5.4: NanoSIMS analysis: $^{15}N/^{14}N$ distribution within symbiotic *Symbiodinium* in *P. damicornis* planulae after 8 hrs exposures to ^{15}N labelled *Alteromonas sp*. (A), *Vibrio alginolyticus* (V), Control/unlabelled *Alteromonas* sp. (C); numbers 1-3 mark replicates; HSI of the ratio $^{15}N/^{14}N$, the image is 40 x 40 µm, 256 x 256 pixels; acquisition time was 20 min, scale bar 5µm. The scale ranges from blue (natural abundance) to magenta (increase over the natural value).

After 24 hrs incubation, ^{15}N levels in *Symbiodinium* decreased significantly (Kruskal-Wallis Test, H: 414.8; Hc: 414.8; p=1.88E-87) in *Alteromonas sp*. treated planulae (Fig. 5.5 A1-3, V1-3; Fig. 5.6) but not in planulae treated with *V. alginolyticus*.

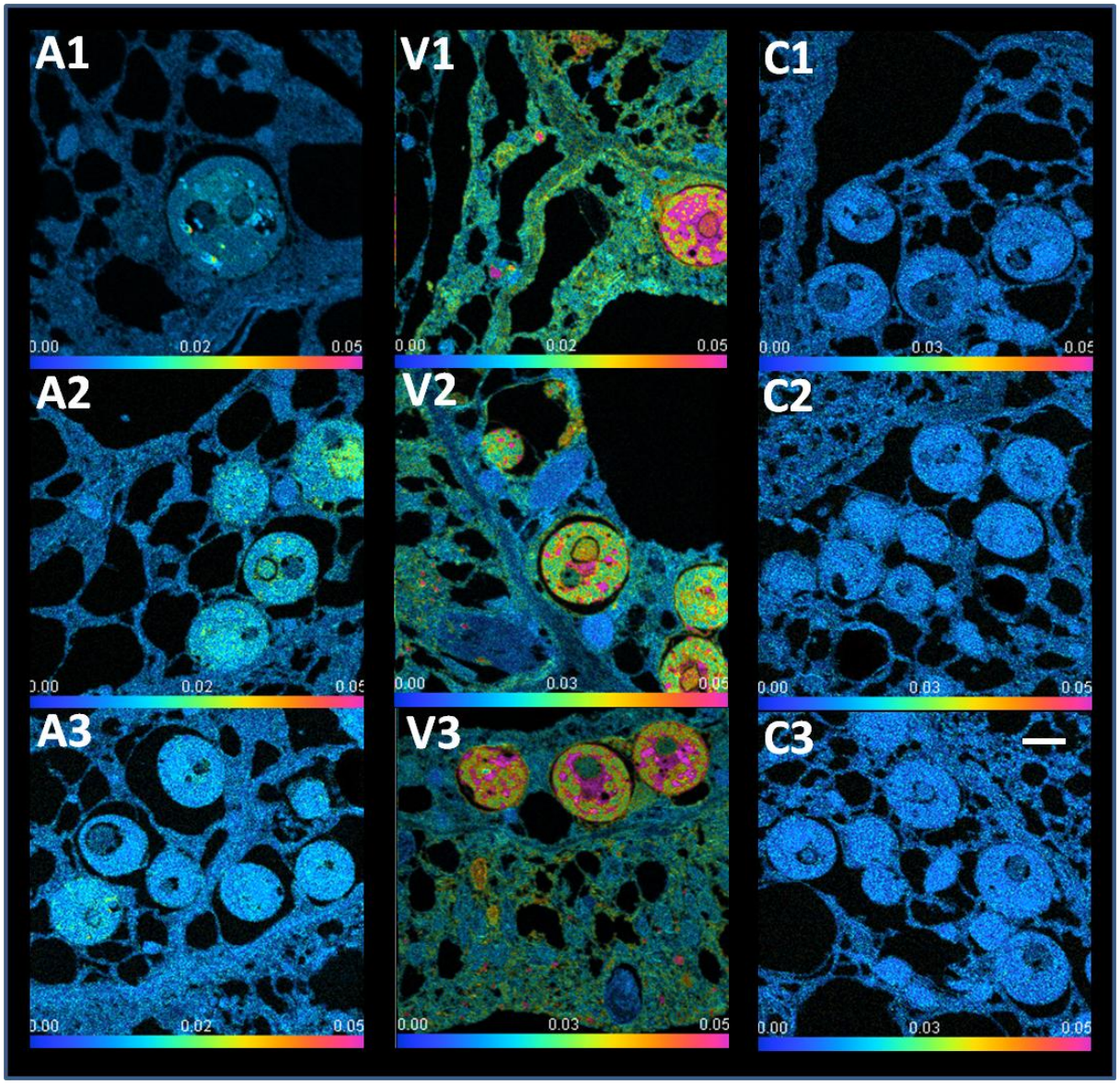


Fig. 5.5: NanoSIMS analysis: $^{15}N/^{14}N$ distribution within symbiotic *Symbiodinium* in *P. damicornis* planulae after 8 hrs exposures to ^{15}N labelled *Alteromonas sp*. (A), *Vibrio alginolyticus* (V), Control/unlabelled *Alteromonas* sp. (C); numbers 1-3 mark replicates; HSI of the ratio $^{15}N/^{14}N$, the image is 40 x 40 µm, 256 x 256 pixels; acquisition time was 20 min, scale bar 5µm. The scale ranges from blue (natural abundance) to magenta (increase over the natural value)

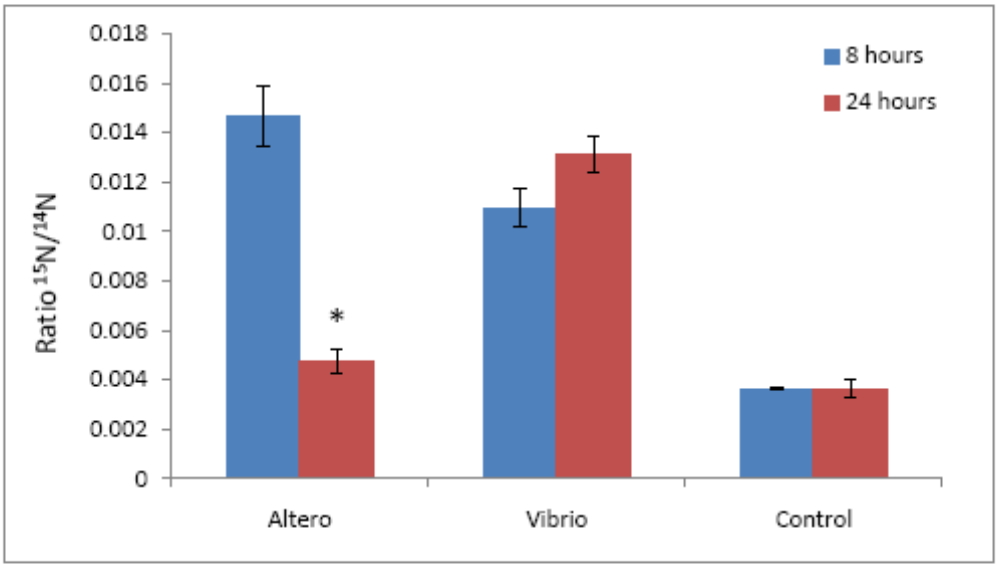


Fig. 5.6: The $^{14}N/^{15}N$ isotope ratio within *P. damicornis* planulae exposed to two different bacteria, *Alteromonas sp*. and *Vibrio alginolyticus* over two time points. Asterisks indicate significant differences between treatments.

In *A. millepora* enriched nitrogen was only found for *V. alginolyticus* treated planulae after 24 hours (Fig. 5.7)

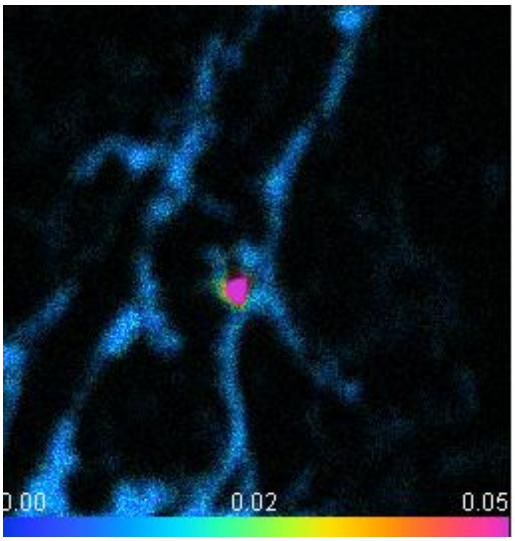


Fig. 5.7 NanoSIMS analysis: Enriched N within the gastrodermis of A. millepora larva after 8 hrs exposure to ^{15}N labelled *Vibrio alginolyticus*. Hue Saturation and intensity scale (HSI) of the ratio $^{15}N/^{14}N$; the image is 40x40µm and 256x256 pixels; acquisition time was 20 min.

Bacterial presence and location within *P. damicornis* planulae were attempted to be visualized by FISH (Fig. S.5.2, supplementary information), using *Vibrio*- and *Alteromonas*-specific fluorochrome-labelled oligonucleotide probes

to co-localize bacteria with enriched nitrogen signals within *Symbiodinium*, however the results were inconclusive and due to time constraints not repeated.

5.5 Discussion

This study demonstrates the ability of coral larvae to take up nutrients which have previously been acquired from the environment by bacteria. The assimilation of nitrogen by bacteria and the passage of nitrogen through the coral planula into the coral-algal symbiont *Symbiodinium* has not been reported or visualized previously and suggests a highly specific functional role of bacteria in the multipartite partnership of the coral holobiont. Symbiotic relationships can range from loose associations to highly specific interactions; endosymbiotic interactions however, are considered highly specific in their function, often represented in diverse grades of adaptations and co-evolution in the host and the symbiont (Kneip et al. 2007). Stable symbiotic relationships between bacteria and free-living dinoflagellates have previously been reported in the dinoflagellate *Alexandrium minutum* which harbours intracellular bacteria (Baker et al. 2003; Palacios and Marin 2008). Likewise an endosymbiotic relationship between the dinoflagellate *Symbiodinium* and the tested bacteria is possible and should be further investigated.

Host cell regulation of microbial populations is an important factor for the stability and success of symbiotic associations. In contrast to *P. damicornis* planulae incubated with *Alteromonas sp*., *Vibrio alginolyticus* treated planulae showed consistent nitrogen enrichment through 24 hours. *Vibrio* species are dominant culturable nitrogen-fixers and several *V. alginolyticus* strains have been suggested to be capable fixing nitrogen (Chimetto et al. 2008). Our observations show that coral larvae are able to take up nitrogen from *V. alginolyticus*; stable ^{15}N levels through the experiment may indicate an establishment of a specific, long-term symbiotic relationship with *V. alginolyticus*,

possibly facilitating *P. damicornis* by fixing and providing nitrogen to corals. *P. damicornis* planulae incubated with *Alteromonas sp.* showed a significant decrease in ^{15}N after 24 hours, suggesting a less specific relationship; bacteria might only transfer but not fix nitrogen in coral larvae. To our knowledge *Alteromonas* bacteria have not been reported to fix nitrogen to date. However, bacteria affiliated with the genus *Alteromonas* have been shown to induce coral settlement (Webster et al. 2004) and one particular strain, isolated in this study induced settlement and metamorphosis in acroporid larvae (Hokoshiki, pers. com.).

A highly specific and close relationship between *V. alginolyticus* and coral planulae is furthermore supported by the observation of *V. alginolyticus* presence in the gastrodermis of *A. millepora* despite the absence of *Symbiodinium*, which seem to be driving the presence of *Vibrios* in coral larvae. In contrast, enriched nitrogen has not been observed in *Alteromonas sp.* treated *A. millepora* incubations. Processes regulating the establishment of mutualistic symbiotic relationships, specific symbiont-host recognition and the question whether they are initiated by the coral host or by bacteria remains unclear.

The finding that enriched nitrogen was only located in the gastrodermis of the aposymbiotic *A. millepora* planulae suggests that nitrogen migrates into the coral planulae via the oral pore and through the gastrodermis into the endodermis where *Symbiodinium* are located. This suggestion is supported by a lack of enriched nitrogen in tissue types other than the outer ectodermis in *P. damicornis* planulae.

The presence of nitrogen in coral mucus cells, derived from bacteria suggests specific interactions between bacteria and coral mucus and an active movement of bacteria towards these cells is likely. The presence of photosynthesizing *Symbiodinium* is strongly correlated with mucus production in corals (Banin et al. 2001) and *Symbiodinium* have been suggested to transfer photosynthates directly to the mucus (Meikle et al. 1988), which acts as an attractant to motile

bacteria (Ducklow and Mitchell 1979). *V. alginolyticus* has previously been reported to display chemotactic responses to algal products including acrylate, dimethylsulfide and glycolate (Sjoblad and Mitchell 1979) and the fact that different *Vibrio* species can fix nitrogen might explain why they are so abundant in the mucus of different coral species (Chimetto et al. 2008).

This Nano SIMS approach opens new avenues to study the complex interactions in the coral holobiont and our study is the first one to report and visualize the onset of specific coral-bacterial interactions in the early and vulnerable life stages of corals. The combined findings for *A. millepora* and *P. damicornis* indicate that coral microbial-associations are established rapidly in brooding corals (< 8 hrs) and that the uptake of bacteria largely increases by the presence of *Symbiodinium* in corals. We demonstrate the bacterial ability to passage nitrogen into the coral *Symbiodinium*, providing additional nutritional support and therefore potentially increasing the productivity and survival in vulnerable early life stages of corals; this is a particularly crucial function in the nutrient poor environments reef-building corals live in.

CHAPTER 6

6.1 Conclusions

Studies investigating the complexity of the coral holobiont have received increasing attention over the last decade. Much of the puzzle is still missing, however, the pieces are coming together and the following discussion will describe and focus on how this work contributed a few pieces to the puzzle.

Previous research has identified diverse and complex bacterial assemblages associated with corals (Sunagawa et al. 2010). These coral-associated communities are distinctly different from microbiota in the sea water (Bourne and Munn 2005) and some coral-species have been reported to associate with specific bacteria (Rohwer et al. 2001; Rohwer et al. 2002). Other studies, including results presented in Chapter 2, have shown environmental parameters to be involved in structuring coral-microbial assemblages rather than species-specificity alone (Hong et al. 2009). Bacterial profiles distinctly changed in three coral species, *Acropora tenuis*, *Favites abdita* and *Pocillopora damicornis* over a study period of one year and bacterial shifts were shown to be influenced by time rather than species. These results indicate that coral-bacterial associations were potentially structured by microbiota in the seawater which again was likely influenced by seasonal, environmental factors such as temperature, benthic communities and oceanographic characteristics. Another interesting observation from this study is the separation of bacterial assemblages between the brooding coral *P. damicornis* and the two broadcast spawners. This separation was ascribed to differing reproductive strategies with spawners releasing gametes once a year while brooders release planulae monthly through summer. If corals acquire suitable microbial partners at a particular seasonal period, bacterial communities will certainly differ between spawners and brooders in times of reproductive activity. *P. damicornis* only displayed similar bacterial

communities with a spawner after coral spawning, which again supports the notion that coral microbiota are influenced by environmental factors. In this example the high input of organic matter due to spawning and subsequent break-down processes enhances microbial activity in the sea water which may have allowed opportunistic colonisation of corals by similar bacteria.

Coral were also collected before and after coral mass spawning to assess if and how a high impact event alters coral-associated microbes and changes were detected for all coral species; the drivers for these shifts remained unclear though and bacterial groups involved could not be identified sufficiently due to large proportions of unclassified bacteria diluting a limited number of clones retrieved from clone library studies.

A contemporary 454 sequencing approach provided the high resolution needed to investigate further the drivers of coral microbial association coral reproduction. Three coral species, *Acropora tenuis*, *Pocillopora damicornis* and *Tubastrea faulkneri* were examined before and after coral mass spawning (Chapter 3) and additional samples from *P. damicornis* collected after planulation which was temporally separated from coral mass spawning. The results emphasized the importance of investigating coral microbial assemblages at different taxonomical levels; while minimal microbial shifts were observed at the taxonomic level of class, the assessment at genus level confirmed distinct bacterial shifts through coral mass spawning for all corals, independently of reproductive activity. Clear changes in bacterial assemblages were also detected for the brooder after planulation. Together these results identified environmental changes caused by the event of coral spawning as well as actual coral reproduction to structure coral-associated bacterial assemblages. Interestingly, most groups associated with the *α-Proteobacteria* increased in abundance after spawning as well as after planulation, suggesting that specific bacteria are involved in coral reproduction irrespective of reproductive strategies; particularly bacteria affiliated with the *Roseobacter* clade followed this pattern. The coral *Tubastrea faulkneri* which does not associate with *Symbiodinium* and was not

reproductively active at the time of study served as an excellent model organism to assess changes in microbial communities on corals, independently of reproductive activity. Indeed *T. faulkneri* harboured similar bacteria to *A. tenuis*, before spawning but changed dramatically through coral mass spawning. With more than 90% of the most abundant OTUs changing in proportions, *T. faulkneri* was exposed to random and non-specific bacterial colonization, likely driven by the stimulated bacterial community in the sea water following coral spawning. This indicates that microbes might colonize corals as suitable habitats rather than corals actively structuring their microbial partners. Compared to the ahermatypic coral the zooxanthellate coral *Acropora tenuis* displayed stable and conserved bacterial assemblages after coral spawning. Corals likely attract microbes by coral mucus properties however, colonisation might lack stability and consistency if the *Symbiodinium*-derived photosynthesis by-products as a mucus-component are missing.

To assess the release of potentially beneficial bacteria with their offspring, seawater was collected from *A. tenuis* and *P. damicornis* maintained in filtered seawater during coral spawning and planulation, respectively. Adult corals released bacteria with their gametes and planulae which are likely to benefit the offspring in the following settlement and survival processes (Chapter 4). This was largely in accordance with the result that *P. damicornis* planulae harboured similar bacteria to the ones released by adult *P. damicornis* during planulation, which were affiliated with the genera *Alteromonas* and *Vibrio*. Another bacterial type shed by adult corals was again affiliated with the *Roseobacter* clade which was detected to be enhanced in abundance in adult corals after spawning and planulation, respectively. *Roseobacter* has previously been reported as one of the first colonizers in early coral life stages (Apprill et al. 2009). Considering the possibility that corals get weakened and are therefore more susceptible to bacterial colonization through the costly event of reproduction, *Roseobacter* and *Pseudomonas*, which have previously been reported as strong inhibitors of coral pathogens (Nissimov et al. 2009); such associated microbiota may protect corals from invasion by potentially pathogenic bacteria. A similar function might apply to the vulnerable early life stages in corals.

Bacteria affiliated with the genus *Alteromonas* have been shown to induce coral settlement (Webster et al. 2004) and one particular strain, isolated in this study induced coral settlement and metamorphosis in acroporid larvae (Hokoshiki, pers. com.). Preliminary studies conducted within this work investigated the temporal acquisition of *Alteromonas* and *Symbiodinium* in *A. millepora* larvae and suggested that *Alteromonas* might facilitate the uptake of *Symbiodinium* in *A. millepora* larvae, however, further experimentation is needed to confirm this suggestion.

To explore functional roles of bacteria from *P. damicornis* planulae, isolates were grown in medium containing stable isotope labelled ^{15}N as the only nitrogen source. Coral planulae of *P. damicornis* and *A. millepora* were then exposed to each bacterial strain. Using a nanoscale second ion mass spectrometry approach, high concentrations of the stable isotope ^{15}N were detected within the coral algal partner *Symbiodinium* in *P. damicornis* planulae, for both, *Alteromonas* and *V. alginolyticus* co-incubated planulae. *A. millepora* larvae which had not acquired *Symbiodinium* were not observed to carry ^{15}N with the exception of two samples in the gastrodermis, provided by *V. alginolyticus*.

The onset of specific coral-bacterial partnerships was visualized and indicated that coral-microbial associations in brooding corals are established rapidly and that the uptake of bacteria greatly increased with the presence of *Symbiodinium* in corals. This is the first study, demonstrating the ability of bacteria to translocate nitrogen into reef-building corals, likely increasing fitness and establishment of early coral life stages, which is particularly important in the nutrient poor environments corals live in. This approach of combined methodologies opens new avenues to study the complex interactions in the coral holobiont

In conclusion this work reveals that: (1) microbial shifts in adult corals over one year are structured by environmental parameters; (2) brooding and broadcast spawning corals show distinct

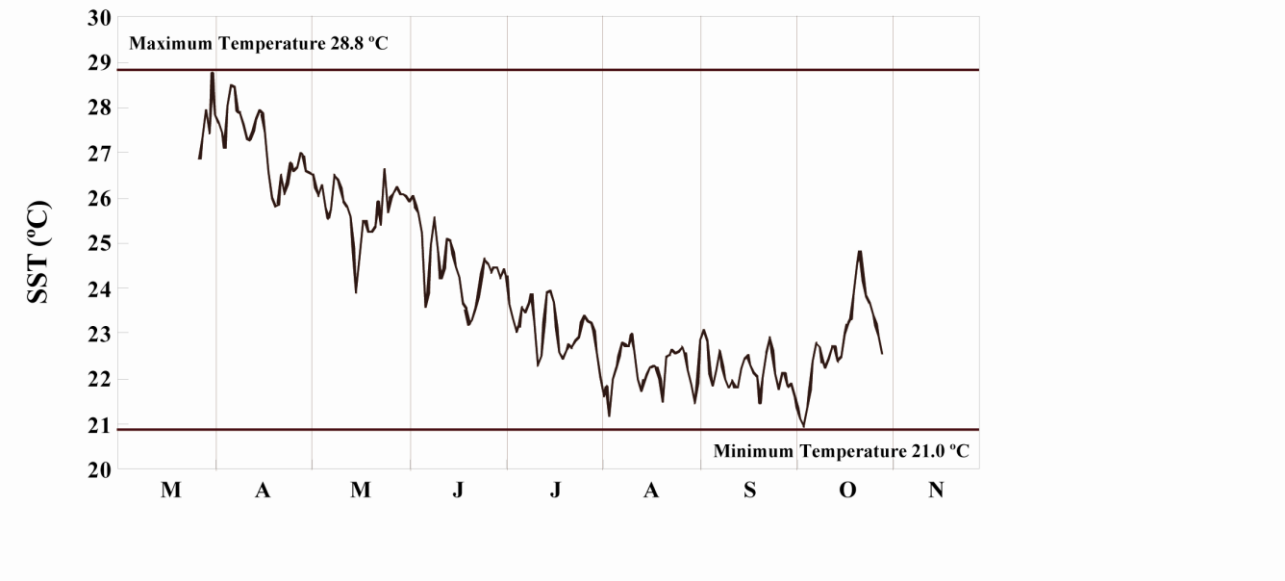
differences in microbial profiles; (3) coral reproduction as well as the environmental effects of coral mass spawning influence changes in coral-microbial communities; (4) particular bacterial groups are released with their offspring in brooders and spawners , which (5) provide benefits – in this case the provision of nitrogen to *P. damicornis* planulae.

6.2 Error discussion

It may be acknowledged that the studies, presented in chapters two, three and four are based on a limited number of replicates and that chapter three and four represents snap-shot studies. Like in most works in this field, a higher number of replicates would have certainly provided a broader context; however the number of samples was limited by permits, logistical constraints and the considerable technical demands, workloads and costs associated with this type of study. Nonetheless, the central questions in this thesis are valid and the results are robust.

SUPPLEMENTARY INFORMATION

CHAPTER 2



Supplementary Figure S.2.1: Daily average sea temperature for the Ningaloo Reef sampling site in 2008.

CHAPTER 5:

S.5.1: Statistical information for Figure 5.5:

	Altero 8	Vibrio 8	Altero 24	Vibrio 24	Control 8	Control 24
Altero 8		0.01761	3.27E-15	0.662	2.28E-26	1.51E-26
Vibrio 8	0.2642		8.93E-17	0.008034	7.18E-33	6.34E-33
Altero 24	4.90E-14	1.34E-15		2.84E-24	2.82E-28	1.83E-28
Vibrio 24	1	0.1205	4.26E-23		5.59E-33	5.59E-33
Control 8	3.43E-25	1.08E-31	4.23E-27	8.39E-32		8.16E-01
Control 24	2.27E-25	9.51E-32	2.74E-27	8.39E-32	1	
Kruskal-Wallis test (H: 414.8; Hc: 414.8; p=1.88E-87) Mann-Whitney pairwise comparison						

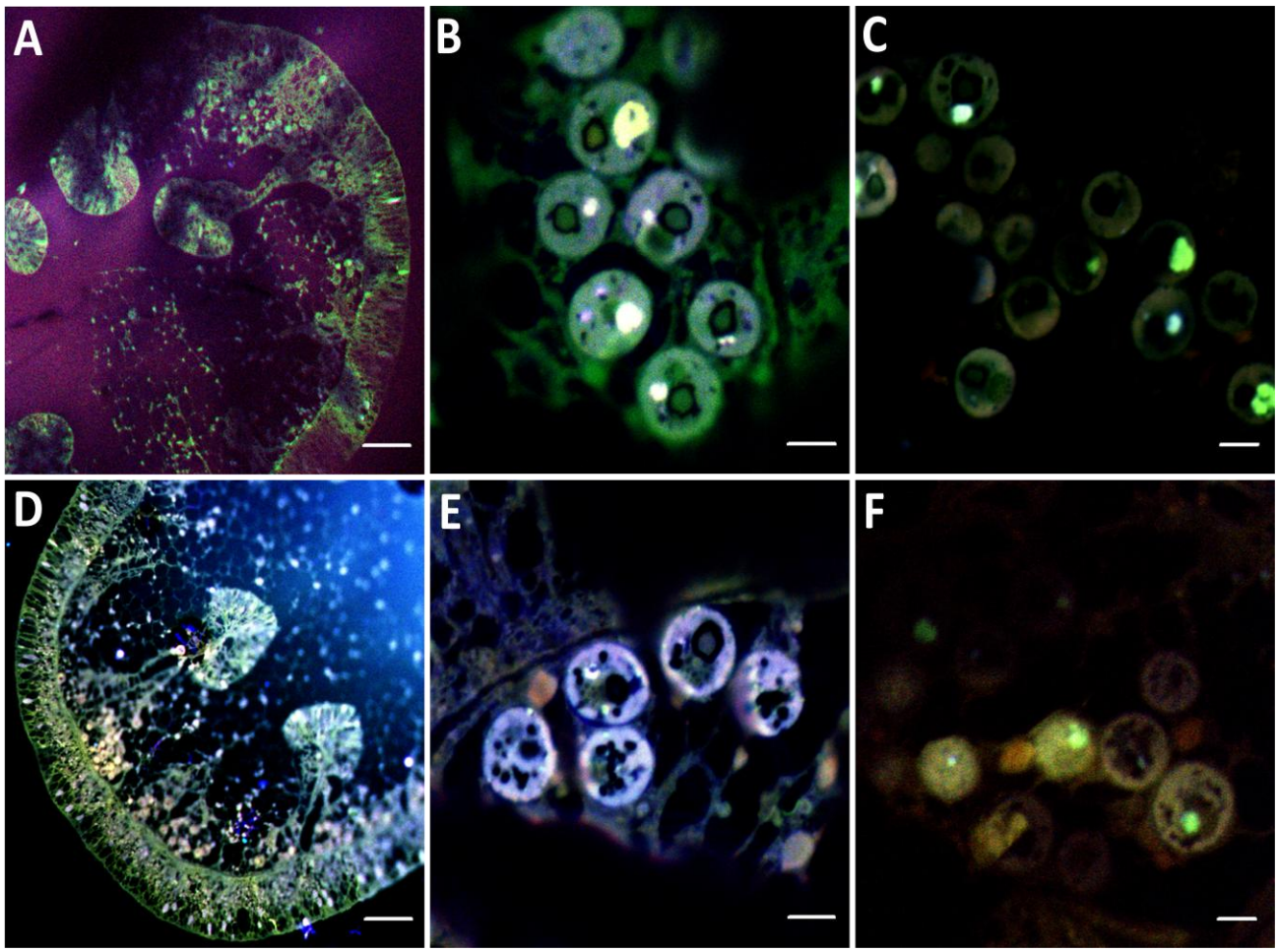


Fig. S.5.2

FISH visualization in *P. damicornis* planulae after 8 hrs exposure to: *Alteromonas sp*. (A) Full section, (B) detail *Symbiodinium*, Probes: Alter2-FLUOS, GAM42a Cy5 double-labelled (d), EUBmix Cy3(d), (C) detail *Symbiodinium*, Probes: NONEUB FLUOS, Cy3, Cy5; *Vibrio alginolyticus* (D) Full section, (E) detail *Symbiodinium*, Probes: Vib572a Cy3, GAM42a Cy5(d), EUBmix FLUOS(d); (F) detail *Symbiodinium*, Probes: NONEUB FLUOS, Cy3, Cy5. All sections are 1 µm in thickness. Scale bar in A and D 50µm, in B, C, E, F 5µm. Pictures are inconclusive; signals could derive from chloroplasts or bacteria.

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