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ABSTRACT

Understanding the drivers of microbiome variation in corals is crucial to better predict the effects of environmental pressures on coral holobionts and coral reef ecosystems. However, much remains to be understood about corals and the interactions they establish with microorganisms. My hypothesis is that the microbiome of the surface mucus layer (SML) is mainly influenced by environmental parameters due to its direct contact with the environment, whereas the tissue microbiome is more driven by the physiology of the coral host. Therefore, the aim of the present work is to distinguish the effect of the host's intrinsic and environmental factors on the microbiome composition in different coral compartments (SML and tissue), and to identify possible overarching trends in the environmental sensitivity of distinct microbiomes within a coral holobiont. Using next-generation amplicon-sequencing of the 16S rRNA gene, the analyses showed that microbiomes of *Acropora* spp. differed significantly between compartments (SML *versus* tissue) and species (*A. tenuis versus A. millepora*), but also among sampling location and season. Seawater samples were characterized by dominance of members of the Synechococcaceae and Pelagibacteraceae. In *Acropora* spp., mucus microbiome was dominated by members of Flavobacteriaceae, Synechococcaceae, Rhodobacteraceae and Pelagibacteraceae families, while the tissue microbiome was dominated by the Endozoicimonaceae family. SML microbiomes of both coral hosts correlated best with environmental parameters as ammonium, total suspended solids, particulate organic carbon, number of raindays and nitrate/nitrite. However, the amount of influence from environmental parameters on the mucus (explaining 12-15% of variation) is relatively low as compared with the influence of those parameters on the seawater microbiome (explaining 49% of variation). In contrast, the tissue microbiomes of the two *Acropora* species

showed distinct and species-specific responses to environmental and physiological parameters, suggesting host-specific modulation of the environmental drivers of the tissue microbiome.

Key-words: Microbial ecology; coral reefs; coral symbionts; bacteria

RESUMO

Os corais são considerados organismos holobiontes, uma unidade viva composta pelo hospedeiro e seu microbioma associado. Os corais, por exemplo, podem estar associados a eucariontes dinoflagelados (família Symbiodiniaceae), procariontes (Bacteria e Archaea) e/ou fungos. Os microrganismos associados aos corais estão envolvidos em diversas funções para o bom funcionamento dos processos fisiológicos do hospedeiro. Por exemplo, podem atuar como mediadores nos ciclos biogeoquímicos e na nutrição do hospedeiro, como também na defesa do mesmo contra agentes patogénicos. Portanto, são essenciais para a manutenção do estado saudável dos corais. Além disso, o microbioma dos corais está sob constante influência de flutuações dos fatores ambientais e fisiológicos dos seus hospedeiros. Compreender os fatores que influenciam a composição e funcionamento do microbioma é crucial para criar estratégias de conservação dos corais e, consequentemente, protegê-los dos efeitos das alterações globais, por exemplo. No entanto, ainda há muito a descobrir sobre a interação simbiótica em corais. O foco da presente tese é a comunidade de procariontes associados, que podem ser referenciados também como comunidades microbianas ou microbiomas ao longo da tese (conforme encontrado na literatura).

As comunidades microbianas podem estar associadas a diferentes partes ou "compartimentos" do pólipo do coral. Os compartimentos incluem o muco superficial, tecido, esqueleto e a cavidade gástrovascular. Cada compartimento possui características únicas e, devido a isto, abriga microbiomas específicos, podendo variar tanto em abundância como em diversidade taxonómica. Por exemplo, estudos revelam que o microbioma do muco superficial de pólipos de corais é mais abundante e mais diverso que o tecido interno dos corais. A camada do muco apresenta-se como uma interface entre o epitélio dos pólipos e a coluna de água. Por isso põe-se a hipótese que a sua comunidade microbiana inclui não só os membros já residentes do muco, como

também transientes originários de outras fontes (tais como a coluna de água, sedimentos em suspensão e/ou organismos bentónicos). Pelo contrário, as camada de tecido (epiderme e gastroderme) dos corais são dominadas principalmente por bactérias do género Endozoicomonas e também por microalgas fotossintéticas (da família Symbiodinaceae), extremamente importantes para a sobrevivência dos corais. Portanto, o estudo específico do microhabitat que o microbioma habita é crucial para o estudo geral de microbiomas de corais. No entanto, atualmente, estudos que englobam fatores que podem influenciar os microbiomas não se focam em diferentes compartimentos, mas apenas num só compartimento (geralmente o tecido). As primeiras descobertas neste tema mostraram que as comunidades microbianas específicas de corais são relativamente estáveis numa escala espacio-temporal. Estudos mais recentes propõem inúmeros fatores, para além da especificidade, que podem afetar a estrutura e a abundância relativa de membros dos microbiomas de corais. Por exemplo, diferenças geográficas, mudanças sazonais, poluição ou o estado fisiológico do hospedeiro. Porém, este tópico ainda é considerado bastante limitado e controverso, e mais estudos são necessários neste contexto..

O principal objetivo deste estudo é distinguir os efeitos de fatores ambientais e fisiológicos do hospedeiros na variação da composição das comunidades microbianas de diferentes compartimentos nos pólipos de corais (muco e tecido), e também, identificar possíveis tendências da sensibilidade ambiental dos microbiomas identificados. A minha hipótese é que o microbioma do muco é principalmente influenciado por parametros ambientais, devido ao seu contato mais direto com o ambiente, enquanto que o microbioma do tecido responde mais às mudanças fisiológicas do hospedeiro. Para testar esta hipótese, dados de Sequenciamento de Nova Geração (NGS) do gene 16S do RNA ribossomal dos microbiomas de diferentes compartimentos (muco e tecido) e espécies de coral (*A. tenuis* e *A. millepora*) foram usados em conjunto com dados de fatores ambientais e fisiológicos dos hospedeiros. O microbioma da coluna de água foi usado como

referência no estudo. Em geral, os resultados mostraram que os microbiomas da *Acropora* diferiram significativamente entre os compartimentos e espécies, e também foram influenciados temporalmente e espacialmente. A riqueza de zOTU ("zero-radius OTU" ou unidade taxonómica operacional, a qual é designada como a menor entidade taxonómica existente) entre os microbiomas analisados diferiu significativamente entre o microbioma do muco, tecido e coluna de água, e também entre as espécies de *Acropora*. A riqueza de zOTU não diferiu entre estações ou local de coleta. O microbioma da coluna de água abrigou a comunidade mais rica, seguido da do muco e da comunidade do tecido. A diversidade alfa baseada no índex de Shannon também diferiu significativamente entre os microbiomas do muco, tecido e coluna de água, porém não diferiu entre espécies de corais, estação e local de coleta. A diversidade de zOTU foi significativamente maior no microbioma do muco em relação ao microbioma da coluna de água, enquanto que o microbioma do tecido foi o menos diverso entre todos. As amostras da coluna de água foram caracterizadas pela dominância de membros de Synechococcaceae e Pelagibacteraceae. Membros pertencentes às famílias Flavobacteriaceae, Synechococcaceae, Rhodobacteraceae e Pelagibacteraceae foram dominantes no microbioma do muco das espécies de *Acropora*, e membros da família Endozoicimonaceae foram dominantes no microbioma do tecidos de ambas as espécies. Os microbiomas do muco de ambas espécies de *Acropora* apresentaram maior influência de parâmetros ambientais como amónia, sólidos suspensos totais, carbono orgânico particulado, número de dias chuvosos e nitritos/nitratos. No entanto, a quantidade de influência dos parâmetros ambientais no muco (explicando 12-15% da variação) é relativamente baixa em comparação com a influência desses parametros no microbioma da água do mar (explicando 49% da variação). Por outro lado, os microbiomas do tecido apresentaram respostas distintas entre as espécies de *Acropora* nos parâmetros ambientais e fisiológicos, sugerindo modulação específica do hospedeiro aos fatores ambientais do microbioma do tecido.

Em conclusão, o presente estudo revela que microbiomas presentes em compartimentos fisicamente distintos respondem diferentemente a fatores ambientais e fisiológicos, o que é uma novidade para os estudos de microbiomas de corais e sua dinâmica. Portanto, este estudo esclarece parte do conhecimento limitado e controverso neste contexto, e também estimula o uso de abordagens metodológicas mais holísticas sobre o tema. Sugestões para estudos futuros podem incluir o uso de outras espécies, diferentes das usadas no presente trabalho, e também testes experimentais (não só baseado em correlações) para o fornecimento de uma compreensão mais ampla da variação de microbiomas em corais.

Palavras-chave: Ecologia microbiana; recifes de corais; simbiontes; bactéria.

Chapter 1

INTRODUCTION

1.1 CORAL REEFS

Corals reefs are complex biological structures, whose frameworks are formed by the synthesis and accumulation of calcium carbonate skeletons by hermatypic scleractinian corals (reef-building corals; Allemand et al. 2004). Coral reefs constitute the largest biogenic structures on the planet and are most commonly known from oligotrophic tropical waters (Stanley, 2001). For example, the Great Barrier Reef (GBR), the world's largest coral reef ecosystem, covers approximately 344,400 km^2 in area. The GBR is comprised of almost 3,000 individual reefs and extends over more than 2,000 Km along the coast of Queensland in north-eastern Australia (GBRMP, 2018). Overall, reef ecosystems provide a wide variety of "ecosystem services", including biological and ecological services to other reef organisms, and support to tropical coastal human communities (Moberg and Folke, 1999; Marshall et al. 2018; Elliff and Kikuchi, 2017). For instance, coral reefs function as important spawning, nursery, breeding and feeding areas to a vast number of reef organisms (Moberg and Folke, 1999). Reefs also provide physical protection to the species that live within the reefs and create favourable conditions for the establishment of seagrass beds and mangrove ecosystems (Moberg and Folke, 1999). For human populations, especially those living in tropical coastlines, coral reefs provide a variety of seafood, such as fish, mussels and crustaceans, function as sinks of global atmospheric CO_2 and also offer tourism-related income and shoreline protection, as well as aesthetic and cultural benefits (Elliff and Kikuchi, 2017; Marshall et al. 2018).

Coral reefs represent the pinnacle of marine biodiversity, hosting approximately 25% of all described marine species (Knowlton et al. 2010). The GBR alone is home to over 600

species of coral, 1,625 species of fish, plus tens of thousands of other marine invertebrates (e.g., sponges, anemones, crustaceans, mollusks and echinoderms) and vertebrates (e.g., sharks, rays and marine turtles) (GBRMP, 2018). Despite the great contribution of coral reefs to marine biodiversity, these ecosystems are currently considered as critically threatened worldwide (Hughes et al. 2017; Hoegh-Guldberg et al. 2018). Coral reefs are experiencing massive global declines due to an increase of local pressures, such as degraded water quality or overfishing, as well as global climate shifts, which include elevated sea-surface temperature and ocean acidification (Knowlton et al. 2010; Hughes et al. 2017; Hoegh-Guldberg et al. 2018; Allemand and Osborn, 2019). Therefore, coral reef specialists and stakeholders are currently focused on factors that are crucial for sustaining coral reef ecosystems and reverse the global coral reef crisis (Veron et al. 2009). For instance, the Australian Reef Restoration and Adaptation Program (RRAP), composed of specialists from different leading Australian research universities and institutes, is compromised to create innovative strategies that can be used for large-scale reef restoration and coral adaptation within the GBR (https://www.gbrrestoration.org/home).

1.2 THE CORAL MICROBIOME

Corals are holobionts, comprised of the coral host and microorganisms such as eukaryotic dinoflagellates (family Symbiodiniaceae), prokaryotes (Bacteria and Archaea) and fungi (Bourne et al. 2016). Microorganisms have fundamental roles within the coral holobiont and therefore they are considered essential to maintain the health of corals (Bourne et al. 2016). For example, they are fundamental drivers of biogeochemical cycling within corals (Lema et al. 2012; Rädecker et al. 2015), can support coral nutrition (Palardy et al. 2008; Muller-Parker and D'Elia, 2015) and provide defense against pathogens (Gochfeld and Aeby, 2008; Shnit-Orland & Kushmaro, 2009). The mutualistic relationship corals establish with their

endosymbiotic dinoflagellates (in the family Symbiodiniaceae), also known as "zooxanthellae", is considered a key factor in their evolutionary success in oligotrophic marine environments and is commonly used as coral health indicator (Muller-Parker and D'Elia, 2015). These photosynthetic symbionts inhabiting the tissue of corals (also other invertebrates) are responsible for up to 100% of the coral's energetic requirements, through the translocation of photosynthetic products to their host (Tremblay et al. 2012; Muller-Parker and D'Elia, 2015).

In contrast to the well-characterized relationship between corals and their photosynthetic dinoflagellates (Muscatine, 1980; LaJeunesse, 2002; Frade et al. 2007), only recently the contribution of other microbial members of the coral holobiont has been recognized (Rowher et al. 2002; Bourne and Munn, 2005; Littman et al. 2009; Lema et al. 2012; Sunagawa et al. 2010; Lee et al. 2015; Sharp et al. 2017). In fact, corals are also associated with a vast diversity of prokaryotes (in the Bacteria and Archaea domains), which together with their eukaryotic counterparts (such as Symbiodiniaceae, other protists or fungi), are often referred to as the coral microbiome. However, in this thesis, we will use the term "coral microbiome" to refer exclusively to the prokaryotic communities associated with corals.

The coral microbiome plays a critical role in the health of corals and can affect the overall response of coral reefs to growing environmental pressures (Ainsworth et al. 2010). Recent evidence suggests that coral-microbe associations are more complex than previously assumed, and that coral microbiomes are strongly influenced by many biotic and abiotic factors (Li et al. 2014; Roder et al. 2015; Ainsworth and Gates, 2016; Grottoli et al. 2018). Moreover, the coral microbiome has been proposed as a transient community responsive to the surrounding environment, including a diverse number of factors, such as temperature, nutrient levels or light intensity (Glasl et al. 2019). Hence, studying the composition and functional contribution of microbiomes to the host health provides an important baseline to understand resilience of corals, and can provide future knowledge to sustain new strategies for coral reef

conservation. However, the precise contribution of the microbiome on the health and resilience of corals still remains poorly understood. Understanding the complexity of coral microbiomes is essential to evaluate the role of microbial communities in coral reef ecosystems.

Studies have classified the prokaryotic microbiome into different categories depending on their specificity and prevalence. For instance, Hernandez-Agreda et al. (2016) suggests an approach in which three functionally distinct sub-communities are present within corals: (i) the core microbiome, composed by a ubiquitous and stable community; (ii), an individual microbiome consistent within specific environmental regimes; and (iii) a more dynamic sporadic community responsive to biotic and abiotic fluctuations. Recent studies revealed that the coral microbiome is particularly dominated by the bacterial phyla Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria and Firmicutes. The classes Gammaproteobacteria, and Alphaproteobacteria showed to be the most dominant within Proteobacteria, and *Endozoicomonas* was the most abundant genus in the coral microbiome (Ainsworth et al. 2015; Hernandez-Agreda et al. 2016; Bourne et al. 2016; Hernandez-Agreda et al. 2017; Weiler et al. 2018; Dunphy et al. 2019).

1.3 CORAL MICROHABITATS

Different compartments of a coral include the coral surface mucus layer, the tissue, the skeleton and the gastric cavity. Evidence has shown that the abundance of persistent microbial taxa can substantially vary among these coral compartments (Sweet et al. 2011). Each compartment has a unique set of features and harbours a specific microbial community (Sweet et al. 2011; Engelen et al. 2018; Pollock et al. 2018; Weiler et al. 2018). Therefore, it is crucial to be specific and determine the microhabitat these microorganisms reside in when studying the coral microbiome.

The aim of the present study is to understand microbiome variation in response to environmental and host physiology parameters over time. As the community inhabiting the surface mucus is in more direct contact with the surrounding seawater, I suggest that change in the mucus-associated community respond to environmental fluctuations, while the tissue-associated community respond to variation in the coral physiology and its associated photosymbionts (the zooxanthellae). Therefore, and unlike most microbial studies focusing only on a single coral compartment or even on the whole animal as a bulk community (Rowher et al. 2002; Bourne et al. 2008; Ceh et al. 2011; Chen et al. 2011; Ainsworth et al. 2015), this work will study microbiome variation in two coral compartments: the surface mucus layer and the coral tissue (Figure 1.1).

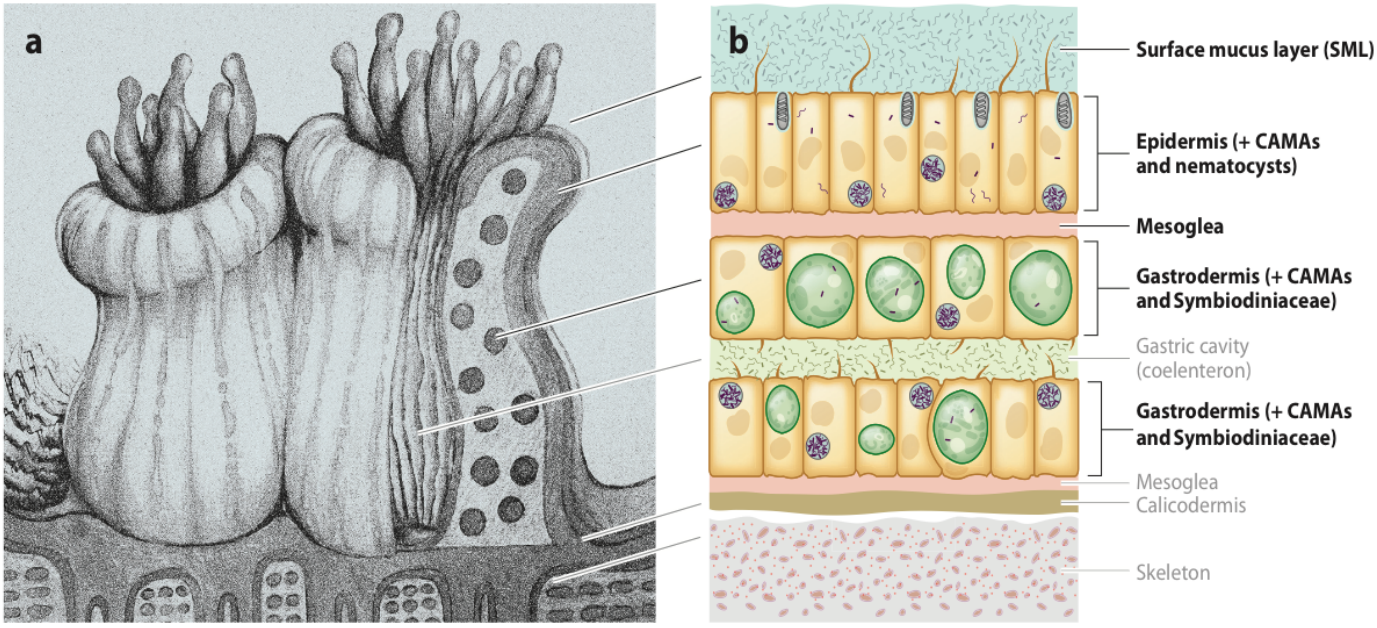

Figure 1.1 (a) Schematic illustration of a coral polyp, and (b) its detailed microstructure representing microhabitats, including surface mucus layer (SML), tissue layers (epidermis, mesoglea, gastrodermis), gastric cavity (coelenteron), and skeleton. The SML and tissue layers are highlighted in bold to represent the microhabitats targeted in the present study. A more abundant bacterial community is represented in the SML as compared to the tissue layers. Coral nematocysts are represented as elongated structures in the epidermis. Symbiodinaceae is represented as large green cells in the gastrodermis. Coral-associated microbial aggregates (CAMAs) are present within the epidermis and gastrodermis layers. Adapted from Bourne et al. (2016).

1.3.1 SURFACE MUCUS LAYER

The surface mucus layer (SML) is a polysaccharide-protein-lipid complex secreted by mucocyte cells to the coral's surface layer (Brown and Bythell, 2005). It provides an interface between the coral epithelium and the surrounding seawater environment. The SML provides important biological functions, including protection (against pathogens, ultra-violet radiation, desiccation, pollutants and others) and nutrition roles through mucociliary feeding to their host (Brown and Bythell, 2005; Ritchie, 2006). Moreover, it is also the habitat for a highly diverse microbial community, including resident members and others that are transient and can be trapped by the mucus upon contact with other environmental sources, such as seawater, sediments in resuspension and other benthic organisms (Brown and Bythell, 2005; Guppy and Byhtell, 2006; Sweet et al. 2011). Studies have shown significant differences between the community structure of the SML and other habitats (other coral compartments, seawater and sediment) (Bourne and Munn, 2005; Brown and Bythell, 2005; Sweet et al. 2011; Glasl et al. 2016). For example, Sweet et al. (2011) found six ribotypes related to the genera *Sphingobacterium*, *Shewanella, Roseobacter, Pseudidiomarina* and *Pseudoalteromonas* exclusively in the mucus microbiome of *Acropora* spp., distinct from other coral compartments (coral tissue and skeleton), water column and sediment samples. While the mucus bacterial community can be different from other coral compartments, it can also partially overlap with the tissue, gastrovascular and the seawater communities (Sweet et al. 2011; Engelen et al. 2018). Moreover, Bourne and Munn (2005) showed that the microbial community of mucus samples resembled more the seawater microbial community than the tissue microbiome.

The SML is also subject to a continuous or periodical replacement or "shedding", a physiological mechanism by which the coral is thought to control the abundance of its associated bacterial community by releasing bacteria from its surface, for example under organic matter stress (Bythell and Wild, 2011; Garren and Azam, 2012). This process can vary

among coral species and overtime (Bythell and Wild, 2011). Mucus shedding is suggested to be part of the coral's life strategy as it can support the maintenance of a healthy mucus microbiome and enhance coral reef's resilience under constant sediment resuspension (Ainsworth et al. 2010; Sweet et al. 2011; Garren and Azam, 2012; Glasl et al. 2016). In a recent study, the shifts in the prokaryotic community composition in the SML of *Porites astreoides* were shown to relate to the mucus aging process that precedes periodical sloughing of the entire mucus layer (Glasl et al., 2016). In aged mucus sheets of *P. astreoides*, opportunistic and pathogenic bacteria were prevalent, and after the release of the aged mucus, the prokaryotic community reverted to its original healthy state (Glasl et al. 2016). This example highlights the dynamic and protective nature of the mucus microbiome.

1.3.2 CORAL TISSUE

The coral organism consists of two distinct tissue layers (epidermis and gastrodermis) (Figure 1) and a connective-tissue layer called mesoglea (Muller-Parker and D'Elia, 2015). Each of them contains numerous cell types, whose functions are still not well determined (Tresguerres et al. 2017). Moreover, the coral tissue provides to the zooxanthellae a protective environment and the inorganic compounds they need for photosynthesis. The products of photosynthesis include sugars, lipids and oxygen that are used by the coral's polyps for growth, cellular respiration, and to enhance the calcification needed to build reefs (Muller-Parker and D'Elia, 2015).

Furthermore, genetic evidence has revealed divergent and diverse genera within the zooxanthellae, or family Symbiodiniaceae (LaJeunesse et al. 2018). The family Symbiodiniaceae is divided in seven formally recognized genera (formerly clades A-G), of which four (formerly clades A-D, now genera *Symbiodinium*, *Breviolum*, *Cladocopium* and *Durusdinium*, respectively) are commonly found in reef-building corals (Muller-Parker and

D'Elia, 2015; LaJeunesse et al. 2018). Symbiodiniaceae diversity differs in different parts of the world. For instance, in the Caribbean, all four genera are found associated to corals, while in the Indo-Pacific Ocean only clades C and D are found (Berkelmans and van Oppen, 2006; Császár et al. 2010; Muller-Parker and D'Elia, 2015). However, interpretation of Symbiodiniaceae ecological distribution patterns is complicated as this is an extreme case of functional diversity and likely co-diversification with the coral host. Each coral lineage holds a very specific number and type of zooxanthellae species and each of them has specific capabilities in terms of photosynthetic performance (Muscatine et al. 1998; Frade et al. 2008a; Frade et al. 2008b; Scheufen et al. 2017).

Coral tissue microbiome is mostly represented by bacterial lineages from the phyla Proteobacteria and Actinobacteria (Bayer et al. 2013; Ainsworth et al. 2015; Engelen et al. 2018). In respect to its characteristics, the bacterial community within the coral tissue is, generally less abundant and less diverse than the one inhabiting the mucus layer (Bourne and Munn, 2005; Koren and Rosenberg, 2006). This is probably a consequence of a more stable medium within the coral tissue as compared to the SML (Bourne and Munn, 2005). *Endozoicomonas* species from the family of Hahellaceae are very abundant bacteria of the coral's endodermal tissue and part of the core coral microbiome (Bayer et al. 2013; Neave et al. 2016; Neave et al. 2017; Glasl et al. 2019). Different coral species seem to harbour specific *Endozoicomonas* (Bayer et al. 2013; Ziegler et al. 2016), which have been associated with the geographic region where the host is present (Ziegler et al. 2016; Neave et al. 2017). Furthermore, higher abundance of *Endozoicomonas* has been correlated with a healthy condition of the host as compared to corals under some type of stress (Glasl et al. 2016; Apprill et al. 2016; Lema et al. 2012; Ziegler et al. 2016). For example, Ziegler et al. (2016) observed a significant decrease in the dominant microbes belonging to the Endozoicomonaceae in two

coral species (*Pocillopora verrucosa* and *Acropora hemprichii*) at sites impacted by increased sedimentation and local sewage.

1.4 FACTORS INFLUENCING THE CORAL MICROBIOME

Seminal studies on the coral microbiome have shown that some bacteria form relatively stable species-specific communities over space and time (Frias-Lopez et al. 2002; Rohwer et al. 2002; Bourne et al. 2008). However, recent studies have proposed a number of factors other than host specificity that can influence the microbiome structure and the relative abundances of individual members of the coral microbiome. There are many possible spatial and temporal drivers of the fluctuation of coral-associated bacterial communities, such as geographic differences (Hong et al. 2009; Littman et al. 2009), seasonal changes (Koren and Rosenberg, 2006; Hong et al. 2009; Ceh et al. 2011; Chen et al. 2011), coastal pollution (Klaus et al. 2007), or physiological status of the host (Littman et al. 2009; Grottoli et al. 2018)

Hong et al. (2009) showed that combined factors are critical to the bacterial composition of *Stylophora pistillata* microbiomes at the southern tip of Taiwan. The study proposed that season, geography and individual physiology of the host all contribute as drivers of the coral microbiome. Littman et al. (2009) presented a similar observation, where the dominant members of the coral microbial community of three species of *Acropora* were mostly defined by the location inhabited by the hosts, rather than by their species affiliation. In contrast, Grottoli et al. (2018) showed microbiome differences between distinct coral species exposed to the dual stress of elevated seawater temperature and ocean acidification. The study proposes that temperature-tolerant corals (*Turbinaria reniformis*) have a more stable microbiome as compared to thermally-sensitive coral holobionts (*Acropora millepora*), suggesting that the type of Symbiodiniaceae associated to the coral host plays a decisive role in the composition and diversity of the microbial community. This also corroborates the idea that coral hosts

having the most stable Symbiodiniaceae community over depth also have the most stable prokaryotic community (Glasl et al. 2017). In summary, an assortment of as many different factors as possible should be considered to obtain a more complete understanding of the association between corals and their microbiomes.

Although studies often focus on only one coral compartment or on the bulk microbial community inhabiting the whole animal (e.g., mucus, tissue and gastrovascular cavity altogether), a recent study analysed simultaneously the effect of environmental and physiological factors on the microbiome of different coral compartments. Pollock et al. (2018) showed, across a number of coral species, that environmental factors (e.g., depth and temperature) had a stronger influence on mucus microbiomes as compared to tissue or skeleton microbiomes, while the coral species and their functional traits were stronger drivers of tissue and skeletal microbiomes relative to mucus microbiomes. The present study has similar objectives, where was analysed the response of coral mucus and tissue microbiomes to a diverse range of environmental and host parameters in two species of *Acropora* (*A. tenuis* and *A. millepora,* Figure 1.2).

The genus *Acropora* is the largest genus of reef-building corals of the Indo-Pacific, and its members are among the most widespread and environmentally sensitive corals, especially to bleaching events (Loya et al. 2001; Shinzato et al. 2011; Hoogenboom et al. 2017). *A. tenuis* and *A. millepora* occupy relatively similar habitats in light-exposed areas of inshore reefs in the GBR and commonly distributed in the western and central Indo-Pacific. Furthermore, both species are classified as Near Threatened (NT) on the IUCN Red List (IUCN, 2008a; IUCN, 2008b). Based on this, it is interesting to understand whether the (spatial-temporal) variation of coral-associated microbiomes differs between distinct but closely related coral species.

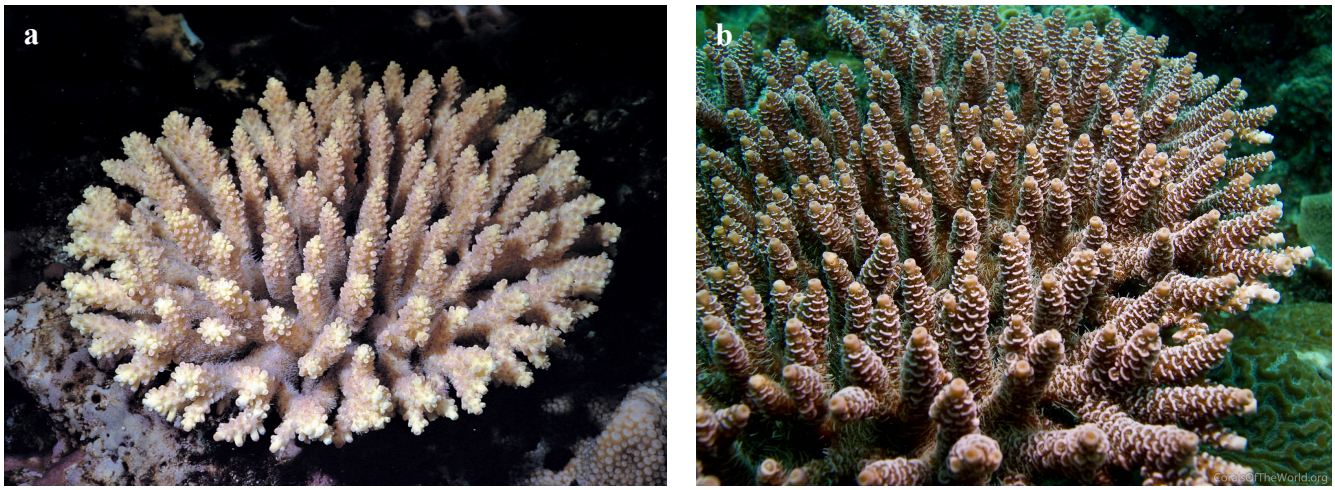

Figure 1.2 (a) *Acropora tenuis* (Veron et al. 2016a)*;* (b) *Acropora millepora* (Veron et al. 2016b).

1.5 METHODS TO STUDY THE CORAL MICROBIOME

Classic cultivation-based methods to study microbial communities have known limitations due to the small number of microbial species that can be cultivated, which are often found to be rarely numerically abundant or functionally significant in natural assemblages (Ngom and Liu, 2014). Therefore, the introduction of culture-independent methods, such as sequencing methods, brought new advances to the study of microbiome compositional and functional diversity (Wagner et al. 1993; Cooney et al. 2002; Yokouchi et al. 2006). In particular, the development of next-generation high-throughput sequencing techniques has allowed researchers to explore the complexity of host-associated bacterial community interactions and dynamics. One of the advantages of these techniques is the generation of a great amount of information (Ansorge, 2009; Cooke et al. 2019). Minor disadvantages are still the overall high cost for generating sequences with very high-throughput and requirements for more efficient hardware due to the huge amount of data to be analysed (Ansorge, 2009; Hernandez-Agreda et al. 2017).

The techniques used for sequence-based microbial profiling can be classified in two methods: the amplicon-based method and whole- metagenomic shotgun method (Cooke et al.

2019). The amplicon-based approach enables researchers to identify organisms based on an amplification of specific genomic regions, such as the 16S ribosomal RNA gene (16S rRNA), commonly used to identify Bacteria and Archaea. Information about microbial abundance, diversity, identification of rare species within and between coral colonies has increased substantially with this technique (Cooke et al. 2019). However, amplicon-based approaches introduce a limitation to the microbiome study. The amplicon-based method is based on an association between the 16S rRNA gene and an operational taxonomic unit (OTU), which is established by clustering sequencing reads that are sufficiently similar to one another and/or to a reference database (Callahan et al. 2017). This can provide compositional data with family or genus resolution, but it is necessarily less precise at the species level (Li, 2015; Rajan et al. 2016). New methods have been developed that circumvent this disadvantage on taxonomic resolution. For example, amplicon sequence variants (ASVs) are the result of a processing methodology that attempts to take into account sequencing error rates and generates sequences with improved resolution (Callahan et al. 2017). Another alternative is the use of whole-genome metagenomics, an untargeted approach to identify the taxonomic composition and also functional potential associated to microbial communities across the entire genomic content present in a sample (Rajan et al. 2016; Quince et al. 2017; Cooke et al. 2019). This can provide a more accurate definition at species level because it uses random primers to sequence overlapping regions of a genome and different databases for taxa classification (Rajan et al. 2016). In order to achieve the coverage and depth needed for species identification, the whole-genome metagenomics method also requires more extensive data and analyses, hence, it is considerably more expensive than the amplicon-based approach (Rajan et al. 2016; Ghosh et al. 2018).

Recent advances in the next-generation sequencing technology are the metatranscriptomics and metaproteomics. Metatranscriptomics aims at characterizing the

expressed genes of microbial communities through analysis of total captured mRNA and provides a functional profile analysis of the community studied (Dubey et al. 2020). With this methodology, it is possible to have a more defined and complete information about the particular community, including not only genetic composition but also functions of a microbiome that are actually transcribed under particular environmental conditions. On the other hand, metaproteomics is focused on the study of all the protein expressed at a given time within an ecosystem (Dubey et al. 2020). This allows the characterization of actually expressed protein functions within microbial communities and can contribute to the identification of novel genes and cellular pathways, as well as recognition of stress-responsive proteins (Maron et al. 2007; Dubey et al. 2020).

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Chapter 2

Journal manuscript

Coupling shifts in the composition of coral tissue- and mucus-associated microbial communities to changes in coral host physiology and environmental fluctuations

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Abstract: Corals associate with diverse bacterial assemblages that show interspecific variability. However, intraspecific spatial and temporal microbial dynamics are far from being understood. These dynamics are relevant because the microbiome plays a fundamental role in the health and stability of coral holobionts, and therefore mediates coral reef responses in the face of climatic stressors. The coral-associated bacterial community varies substantially among coral compartments, however the factors controlling the occurrence, abundance and distribution of bacterial groups have rarely been explored for different compartments simultaneously. Here, we hypothesize that the surrounding environment primarily influences the mucus microbiome, whereas the tissue microbiome responds more to changes in host physiology. we quantified the effect of multiple host and environmental factors on microbiome variation in different coral compartments (mucus and tissue) of two species of *Acropora* (*A. tenuis* and *A. millepora*) common along inshore reefs of the Great Barrier Reef. Next-generation sequencing of the 16S rRNA gene from 226 samples collected over 16 months, revealed significant differences in bacterial richness, diversity and community structure among mucus, tissue and the surrounding seawater. Seawater samples were dominated by members of the Synechococcaceae and Pelagibacteraceae whereas the mucus microbiome of *Acropora* spp. was dominated by members of Flavobacteriaceae, Synechococcaceae, Rhodobacteraceae and Pelagibacteraceae and the tissue was dominated by Endozoicimonaceae. Environmental factors including ammonium, total suspended solids, particulate organic carbon, number of rain days and nitrate/nitrite were the primary drivers of the mucus microbiome of both *Acropora* species. In contrast, the tissue microbiome differed between host species in its response to environmental and physiological factors, suggesting host-specific modulation of the environmental drivers of the tissue microbiome. These results highlight that microbiomes

inhabiting different niches within the coral holobiont differ in their response to host physiology and environmental factors

Key-words: Microbial ecology; coral reefs; coral symbionts; coral compartments

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INTRODUCTION

Corals are considered holobionts, as they are associated with diverse microbial assemblages (Rohwer et al. 2002). Besides the well-characterised symbiotic association with eukaryotic dinoflagellates in the family Symbiodiniaceae*,* corals are also associated with a vast diversity of prokaryotes (Bacteria and Archaea), generally referred to as the coral microbiome (Rohwer et al. 2002; Bourne et al. 2016). The microbiome has a fundamental role in the health and stability of the coral holobiont; it recycles nutrients, removes waste products and can act as defence against pathogens (Morris et al. 2011; Lema et al. 2012; Rädecker et al. 2015; Rosado et al. 2019). Coral microbiome composition, however, can be influenced by a variety of factors. In seminal studies, coral microbiomes were described to be host species-specific and to remain relatively stable over space and time (Frias-Lopez et al. 2002; Rohwer et al. 2002; Bourne et al. 2008). However, recent studies have proposed that spatial-temporal factors such as environmental parameters (Chen et al 2011); geographic differences (Hong et al. 2009; Littman et al. 2009), seasonal changes (Koren and Rosenberg, 2006; Hong et al. 2009; Ceh et al. 2011; Chen et al. 2011), coastal pollution (Klaus et al., 2007), and the physiological status of the host (Littman et al., 2009; Grottoli et al. 2018) can also influence the microbiome structure. Those factors can control the occurrence and relative abundance of bacterial groups. For instance, Li et al. (2015) reported a dynamic relationship between the community structure of coral-associated bacteria and the seasonal variation in environmental parameters, such as dissolved

oxygen and rainfall. Given the importance of microbes in the health of the coral holobiont and their sensitivity to a variety of host-intrinsic and environmental factors, it has recently been suggested that changes in the microbial communities can act as early warning indicator for holobiont stress (Glasl et al. 2017b; Roitman et al. 2018)

The coral animal provides different microhabitats for its microbial associates, including coral surface mucus layer (SML), coral tissue, skeleton and gastrovascular cavity (Sweet et al. 2011; Engelen et al. 2018; Pollock et al. 2018; Weiler et al. 2018). Each microhabitat has a unique set of features and harbours a specific microbial community (Sweet et al. 2011; Engelen et al. 2018; Pollock et al. 2018; Weiler et al. 2018), hence, understanding microhabitat specific associations of the coral microbiome and their sensitivities to environmental fluctuations is crucial to better understand the role of microbes in a coral holobiont. For example, the SML is a polysaccharide-protein-lipid complex and it provides an interface between the coral epithelium and the surrounding seawater (Brown and Bythell, 2005). It has an important functional role for the coral animal including sediment cleansing, nutrition and the physical protection against pathogens, ultra-violet radiation, desiccation and pollutants (Brown and Bythell, 2005; Ritchie, 2006). Studies have shown significant differences between microbial communities associated with the SML and other coral microhabitats or the surrounding seawater environment (Bourne and Munn, 2005; Brown and Bythell, 2005; Sweet et al. 2011; Glasl et al. 2016). For example, Sweet et al. (2011) found six ribotypes related to the genera *Sphingobacterium, Shewanella, Roseobacter, Pseudidiomarina* and *Pseudoalteromonas* exclusively in the mucus microbiome of *Acropora* sp. Although the microbial community composition differs among coral compartments, particular microbiome members found in the coral's SML do overlap with both the tissue and the seawater microbial communities (Sweet et al. 2011).

In contrast to the SML, the coral tissue consists of two distinct tissue layers (epidermis and gastrodermis) and a connective-tissue layer called mesoglea (Muller-Parker and D'Elia, 2015). The coral tissue hosts photosymbiotic dinoflagellates (family Symbiodiniaceae*)*, who in return provide up to 100% of energy required by their coral host (Muller-Parker and D'Elia, 2015). The presence of Symbiodiniaceae communities have also been shown to influence bacterial communities structure through the release of complex organic molecules such as the organosulfur compound Dimethylsulfoniopropionate (DMSP) and dimethylysulphide (Bourne et al. 2013). Coral tissue microbiome is mostly represented by bacteria belonging to the phyla Proteobacteria and Actinobacteria (Bayer et al. 2013; Ainsworth et al. 2015; Engelen et al. 2018). *Endozoicomonas* belonging to the family Hahellaceae are very abundant bacteria of the coral's endodermal tissue and part of the core microbiome (Bayer et al. 2013; Neave et al. 2016; Neave et al. 2017; Glasl et al. 2019). When compared to the SML, the prokaryotic community in the tissue is significantly less dense and less diverse (Bourne and Munn, 2005; Koren and Rosenberg, 2006), and it has been hypothesised that it is probably associated to a more spatially stable and host controlled environment (Bourne and Munn, 2005).

In this study, we test the hypothesis that different coral compartments (SML and tissue) of *Acropora* spp. harbour distinct microbial communities and that microbiome dynamics within these microhabitats are driven by a different set of factors. We hypothesize that the SML microbiome is mainly influenced by environmental parameters due to its direct contact to the surrounding seawater environment, while the tissue microbiome responds more to the changes in the physiology of the host. Therefore, the aim of the present work is to distinguish the effect of host and environmental factors on the microbiome composition in different coral compartments (SML and tissue), and to identify possible overarching trends in the environmental sensitivity of distinct microbiomes within a coral holobiont.

MATERIAL AND METHODS

Field collections, immediate sample processing, and DNA extractions for 16S rRNA gene sequencing were performed by PhD candidate Bettina Glasl at the Australian Institute of Marine Sciences (AIMS, Townsville, Australia). Physiological assays and respective sampling pre-processing, handling of next-generation sequencing output data and environmental metadata, and statistical analyses were performed by the author of this thesis project at AIMS between October 2018 and May 2019.

2.1 Sample collection

Samples of *Acropora millepora* (Ehrenberg, 1834)*, Acropora tenuis* (Dana, 1846) and seawater were collected for 16S rRNA gene sequencing, along with environmental metadata, between February 2016 and May 2017. The period was divided in two seasons: from May to October, classified as dry or winter season and from November to April as wet or summer season according to the Bureau of Meteorology of the Australian Government (Commonwealth of Australia, 2019). The sampling was performed at three sites in the Great Barrier Reef at monthly (Magnetic Island - Geoffrey bay) and periodic (Orpheus Island – Channel and Pioneer bay) intervals (Figure 2.1). All samples were collected under the permit G16/38348.1 issued by the Great Barrier Reef Marine Park Authority.

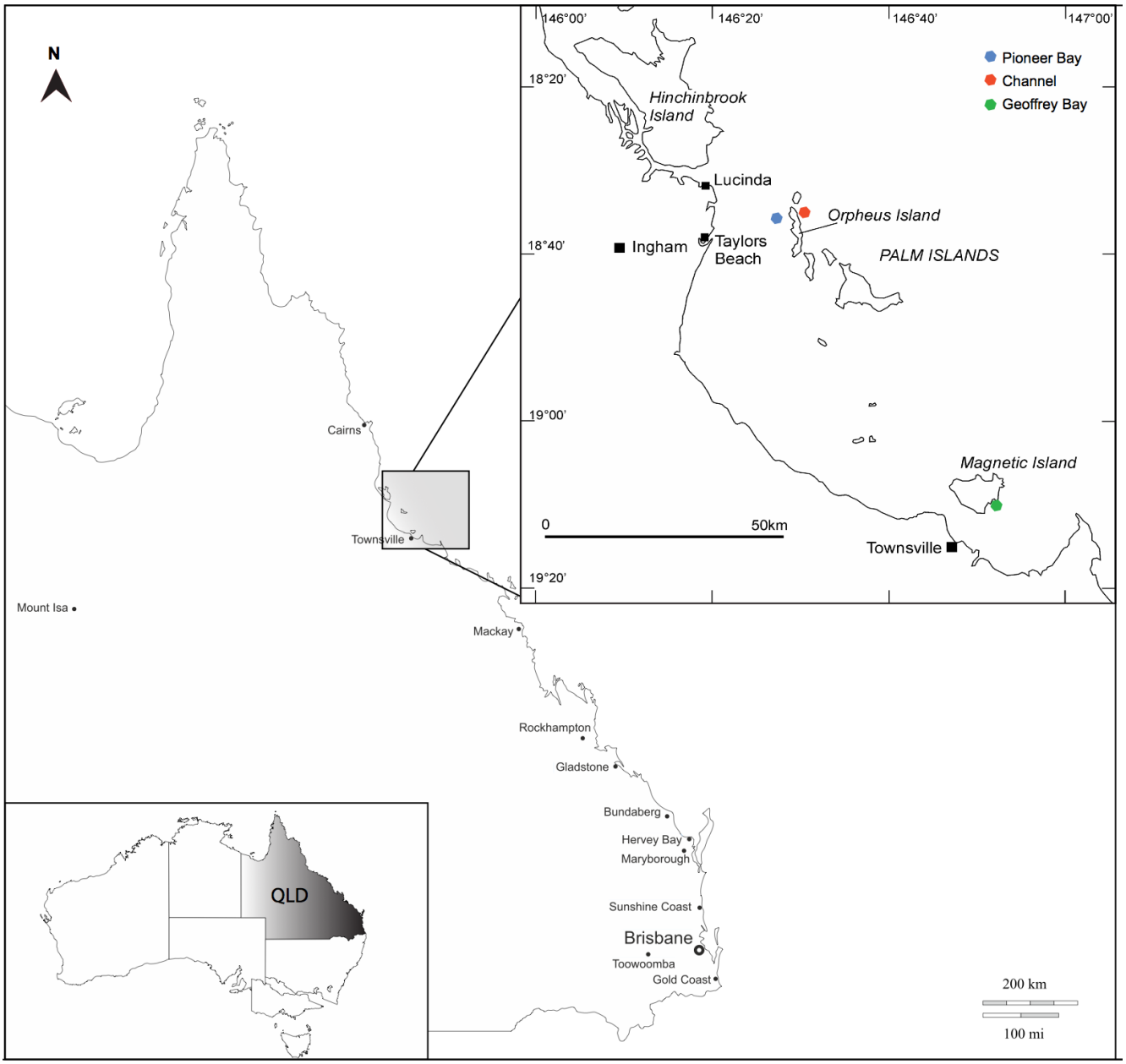

Figure 2.1 Map showing the sampling locations at Magnetic (Geoffrey Bay) and Orpheus (Pioneer Bay and Channel) Islands, Australia. Adapted from Davis et al. (2004)

Samples (n= 3/ sample type/ sampling event) for molecular analysis were collected following the standard operational procedures of the Australian Marine Microbial Biodiversity Initiative (AMMBI) as previously described by Glasl et al. (2019b). In brief, fragments (branches) of both coral species were collected, rinsed with 0.2 µm-filtered seawater and placed into cryogenic vials for further extraction of the tissue. The coral surface mucus layer from the

same fragments was collected with sterile cotton swabs as previously described by Glasl et al. (2016). Seawater samples for molecular analysis were collected in sterile collapsible bags, pre-filtered through a 50 µm filter mesh to remove large particles and subsequently filtered onto a 0.2 µm Sterivex filter (Millepore). All samples were immediately snap frozen in liquid nitrogen after collection and stored at -80°C until further processing. To acquire environmental information, water and sediment samples were collected in duplicate for each sampling event and further analysed according to the standard procedures of AIMS. Seawater temperatures were obtained from AIMS long-term monitoring temperature records.

2.2 Immediate sample preparation and genetic assays

The frozen coral tissue kept in -80°C was airbrushed into a *ziploc* bag with constant Phosphate-buffered saline (PBS) solution added until all tissue was removed from the skeletal fragment. The resultant slurry was then homogenized for 1 min at 12,500 rpm using a hand-held tissue homogenizer (Heidolph Silent Crusher M), pelleted (10 min at 16,000 rcf) and snap frozen in liquid nitrogen. DNA from the tissue and mucus samples was extracted using the DNeasy PowerBiofilm kit (QIAGEN). DNA extracts were sent on dry ice to the Ramaciotti Centre for Genomics (Sydney, Australia) for sequencing. The bacterial 16S rRNA gene was sequenced using the 27F (Lane, 1991) and 519R (Turner et al. 1999) primers on the Illumina MiSeq platform using a dual indexed 2 x 300 bp paired-end approach.

2.3 Sequence analysis

Sequencing data were analysed as single nucleotide variants following the standardised platform of the Australian Microbiome Initiative (Brown et al. 2018). In brief, paired-end reads were merged using FLASH software (Magoc & Salzberg, 2011) and FASTA formatted sequences were extracted from FASTQ files. Sequences <400 bp in length and / or containing

N's or homopolymer runs of >8 bp were removed with MOTHUR (v1.34.1) (Schloss et al. 2009). Sequences were de-replicated and ordered by abundance using USEARCH (64 bit v10.0.240) (Edgar et al. 2010). Sequences with less than 4 representatives and Chimeras were removed, and the quality-filtered sequences were mapped to chimera-free zero-radius operational taxonomic units (zOTUs). A table containing the samples and their read abundances was created and the zOTUs were taxonomically classified with SILVA v132 database (Yilmaz et al. 2014) using MOTHUR's implementation of the Wang classifier (Wang et al. 2007) and a 60% Bayesian probability cut-off.

Chloroplasts and mitochondria derived reads were removed from the dataset and remaining data was rarefied to a sequencing depth of 3,500 reads per sample in R (R Development Core Team, 2015) using subset_taxa () function in the phyloseq package (McMurdie and Holmes, 2013). The abundance of reads per sample was transformed into relative abundances (in the phyloseq package, McMurdie & Holmes, 2013).

2.4 Physiological trait assays

For spectrophotometric photo-pigment and protein quantification, measurements of absorbance were done in triplicate in a Cytation 3 multi-mode microplate reader (BioTek, Winooski, USA) and the analysis using the software Gen5 (BioTek, Winooski, USA). All physiological parameters measured were normalized to the volume of the tissue homogenate.

2.4.1 Pigment quantification

To determine chlorophyll concentration, the tissue pellet was thawed on ice to avoid sample degradation and then resuspended in 1 ml of 90% ethanol. The samples were sonicated for 1 min and centrifuged for 5 min at 10,000 g. Subsequently, 700 µl of the supernatant was removed and transferred to a new tube. The resuspension, sonication and centrifugation were

repeated on the remainder of the pellet. The supernatant was recovered again, combined with the previous extraction and mixed by inversion.

Sample extract and 90% ethanol (blank read) were loaded in triplicate (200µl each) to a 96-well plate and the absorbance was recorded at 470, 632, 649, 665, 696 and 750 nm. After correcting all absorbance measurements with those obtained in blank runs, the pigments were calculated using the following equations, where E = Absorbance at the specified wavelength (nm). (Ritchie, 2008; Lichtenthaler, 1987):

$$\boldsymbol{Chlorophyll\ a}\ \left(\frac{\mu g}{mL}\right) = \frac{[(-0.9394*(E632-E750))+(-4.2774*(E649-E750))+(13.3914*(E665-E750))]}{0.496}$$

$$\boldsymbol{Chlorophyll\ b}\ \left(\frac{\mu g}{mL}\right) = \frac{[(-4.0937*(E632-E750))+(25.6865*(E649-E750))+(-7.3430*(E665-E750))]}{0.496}$$

$$\boldsymbol{Chlorophyll\ c}\ \left(\frac{\mu g}{mL}\right) = \frac{[(28.5073*(E632-E750))+(-9.9940*(E649-E750))+(-1.9749*(E665-E750))]}{0.496}$$

$$\boldsymbol{Chlorophyll\ d}\ \left(\frac{\mu g}{mL}\right) = \frac{[(-0.2007*(E632-E750))+(0.0848*(E649-E750))+(-0.1909*(E665-E750))+(12.1302*(E696-E750))]}{0.496}$$

$$\boldsymbol{Total\ Chlorophyll}\left(\frac{\mu g}{mL}\right) = \frac{[(24.1209*(E632-E750))+(11.2884*(E649-E750))+(3.7620*(E665-E750))+(5.8338*(E696-E750))]}{0.496}$$

$$\boldsymbol{Total\ Carotenoids}\ (\boldsymbol{\mu g/mL}) = [\frac{\left((1000*\frac{E470-E750}{0.496})-(2.13*Chla)-(97.64*Chlb)\right]}{209}$$

2.4.2 Protein quantification

To quantify the total protein content in the coral host tissue samples, a commercial colorimetric protein assay kit was used with bovine serum albumin (BSA) as standard (Pierce BCA Protein Assay Kit).

The tissue pellet was thawed on ice and resuspended in 1 ml PBS. 25µl of the resuspension was added to 200 µl of working reagent from the kit in a 96-well plate. The plate was mixed thoroughly on a plate shaker for 30 seconds and then incubated at 37°C for 30 min. The plate was cooled down at room temperature. The absorbance was measured at 563 nm. The measurements of the standards and samples were also corrected with those obtained in blank runs to remove background absorbance. For each plate, a protein standard curve was obtained

using BSA solution at concentrations between 25 and 2,000 µg ml^{-1}. The total protein concentration within each sample was calculated using the standard curve.

2.4.3 Symbiodiniaceae cell counting

To determine symbiont cell concentrations in the coral tissue, the tissue pellet was thawed on ice, resuspended in 1 ml of 0.2 µl filtered seawater and added to 1 ml of 10 % formalin to preserve the symbiont cells. The solution was passed through a syringe needle to reduce cell agglomeration and diminish the bias from cell clumps.

Samples were then mixed for 1 min and 10 µl of the homogenate was loaded onto a Neubauer haemocytometer (0.100 mm depth). Zooxanthellae cells were counted under 40 x magnification with an Olympus CX31 light microscope. In total, 24 haemocytometer squares (each with 0.1 µL volume) were used per sample to ensure robustness of density determinations.

Zooxanthellae density in the coral tissue homogenate was determined by the following equation:

$$\frac{Number\ of\ cells}{ml} = \frac{Total\ number\ of\ cells}{Number\ of\ squares} * Dilution\ Factor * 10{,}000$$

2.4.5 Statistical analyses

Statistical analyses were performed using RStudio Version 1.1.463. Alpha- and beta-diversity analysis of microbial communities were performed on relative abundance data at zOTU level. zOTU richness and Shannon-Weaver diversity were compared across host compartments, host species and reference seawater samples using Analysis of Variance (ANOVA). Non-Metric Multidimensional Scaling (NMDS) was used to illustrate the microbial community structure among host species and host compartments based on Bray-Curtis dissimilarities (phyloseq package, McMurdie and Holmes, 2013). Permutational Multivariate Analysis of Variance (PERMANOVA; 9999 permutations) was used to test for differences in

microbial structure among host species, host compartments, season and location using the adonis() function of the vegan package (Oksanen et al. 2013).

Environmental and physiological variables were standardized and checked for collinearity using the Pearson correlation coefficient. Redundant variables based on Pearson's correlation (> 0.7 or < -0.7; Dormann et al. 2013) were removed from the analysis. For environmental samples, Principal Component Analysis (PCA) was used to check for further variable correlation. Afterwards, non-correlated variables were subsequently tested for season and site influence using two- way ANOVA and then used in a Bray-Curtis distance-based Redundancy Analysis (db-RDA), which quantifies the impact of the explanatory variables on the microbiome (dis)similarities (Legendre and Anderson, 1999). zOTU relative abundance, environmental and physiological metadata, seasons and sampling location were used for db-RDA using the phyloseq package (McMurdie and Holmes, 2013). The analysis tests the statistical relationship between microbial community composition and the environmental/physiological variables for each coral compartment and host species. A model selection tool (ordiR2step() function in the vegan package, Blanchet et al. 2008) was performed to select the best db-RDA model (or the best explanatory variables) for the variation in microbiome composition of each coral compartment (mucus and tissue) in each host species (Johnson and Omland, 2004). The significance of each explanatory variable was confirmed with an ANOVA-like permutational test (function "permutest") for dbRDA. The explanatory value (in %) of significant explanatory variables (e.g. environmental and physiological parameters, season and sampling location) on each microbiome was assessed with Variation Partitioning Analysis of the vegan package (Oksanen et al. 2013)"

To conclude, a correlation matrix between taxa abundance and the chosen variables was generated using the R package MicrobiomSeq (Ssekagiri et al. 2017). The correlation matrix

showed the relationship between the 20 most abundant families and the chosen variables based on Pearson correlation (default) using a p-value ≤ 0.05.

RESULTS

Coral-associated and seawater microbiome

The bacterial 16S rRNA genes of 226 samples, including coral tissue (n=42 for *A. millepora*; n=48 for *A. tenuis*), coral mucus layer (n=42 for *A. millepora*; n=46 for *A. tenuis*) and seawater (n=48; used as reference samples) were sequenced and 16,117 zOTUs were identified based on single nucleotide variants level.

zOTU richness differed significantly among surface mucus layer, tissue and seawater microbiomes and between the two *Acropora* species (Table 2.1). zOTU richness did not differ significantly between season or sampling location (Table 2.1). Seawater microbiome harbored the richest microbial community (558 zOTU ± 50.1), followed by the mucus (*A. tenuis,* 442 zOTU ± 255; *A. millepora,* 267 zOTU ± 203) and tissue (*A. tenuis,* 147 zOTU ± 122; *A. millepora,* 118 zOTU ± 37) (Table 2.2). Alpha diversity based on Shannon Index also differed significantly among surface mucus layer, tissue and seawater microbiomes, but not among coral species, seasons or sampling locations (Table 2.1). Alpha diversity of mucus samples was slightly (but significantly) higher (Shannon Index: *A. tenuis*, 4.94 ± 0.794; *A. millepora*, 4.50 ± 0.835) than seawater samples (Shannon Index: 4.43 ± 0.192) (Table 2.2). In contrast, the diversity of the tissue microbiome was the lowest (Shannon Index: *A. tenuis*, 3.28 ± 0.796; *A. millepora*, 3.30 ± 0.659) and strongly differed from mucus and seawater microbiomes (Table 2.2).

Table 2.1 ANOVA results for zOTU richness and alpha diversity based on Shannon Index for microbial communities in different habitats (seawater, mucus and tissue), compartments (mucus and tissue), coral species (*Acropora tenuis and A. millepora*), season (summer and winter) and site (Geoffrey Bay, Pioneer Bay and Channel).

Factor	OBSERVED OTUs			SHANNON INDEX		
	df	F	*p-value*	*df*	F	*p-value*
Habitat	2	107.6	**< 0.001**	2	100.1	**< 0.001**
Compartments	1	14.31	**< 0.001**	1	151.32	**< 0.001**
***Acropora* spp.**	1	18.11	**< 0.001**	1	14.31	0.079
Season	1	1.06	0.304	1	1.82	0.178
Site	2	2.271	0.106	2	0.757	0.470

Table 2.2 Post hoc Tukey test for pairwise-comparisons of observed zOTUs and alpha diversity (based on Shannon Index) across microbial habitats.

Diversity proxi	Pairwise Comparison	Difference of means	Adjusted *p*-value	CI (95%)	
				Lower	Upper
Observed OTUs Habitat	Seawater -mucus	199.02	**< 0.001**	128.54	269.51
	Tissue - mucus	-225.32	**< 0.001**	-284.21	-166.43
	Tissue - seawater	-424.35	**< 0.001**	-494.55	-354.14
Shannon Index Habitat	Seawater - mucus	-0.30	**0.049**	-0.60	-0.00002
	Tissue - mucus	-1.44	**< 0.001**	-1.68	-1.19
	Tissue - seawater	-1.14	**< 0.001**	-1.43	0.84

The microbial community of all samples (n=226, including mucus, tissue and seawater) was dominated by sequences attributed to the phyla Proteobacteria (49%), followed in dominance by Bacteroidetes (17%) and Planctomycetes (8%). Figure 2.2 illustrates the

community composition of both coral compartments and seawater samples (including only the most dominant families). Mucus microbiomes for both *Acropora* species were characterized by members of the family Flavobacteriaceae (average relative abundance ± SD; for *A. tenuis*: 16.17 ± 9.11 %; *A. millepora*: 17.28 ± 10.52 %), Synechococcaceae (*A. tenuis*: 10.82 ± 7.41 %; *A. millepora*: 10.78 ± 9.16 %), Rhodobacteraceae (*A. tenuis*: 6.59 ± 6.44 %; *A. millepora*: 6.29 ± 5.92 %) and Pelagibacteraceae (*A. tenuis*: 5.86 ± 6.34 %; *A. millepora*: 4.23 ± 5.04 %) and Endozoicimonaceae (*A. tenuis*: 10.95 ± 18.19 %; *A. millepora*: 1.64 ± 4.16 %). On the other hand, the tissue microbiome was dominated by the Endozoicimonaceae family (*A. tenuis*: 50.05 ± 32.34 %; *A. millepora*: 29.36 ± 26.69 %) and, with much lower abundance proportions, by members of Synechococcaceae (*A. tenuis*: 5.25 ± 6.01 %; *A. millepora*: 11.42 ± 14.15 %) and Rhodobacteraceae (*A. tenuis*: 6.61 ± 12.38 %; *A. millepora*: 7.55 ± 14.08 %). Seawater samples were mostly characterized by members of Synechococcaceae (35.28 ± 5.15 %) and Pelagibacteraceae (20.16 ± 5.41 %), but also by Flavobacteriaceae (8.16 ± 3.26 %) and Rhodobacteraceae (7.47 ± 4.42 %). In general, tissue and mucus microbiomes shared 2,700 zOTU (16.75 %) of all zOTUs found in the study, including members of genera *Trichodesmium*, *Synechococcus* and *Phaeobacter* sp. Mucus and seawater microbiomes shared 2,470 zOTU (15.32 %) and the tissue and seawater shared 1,477 zOTU (9.16 %).

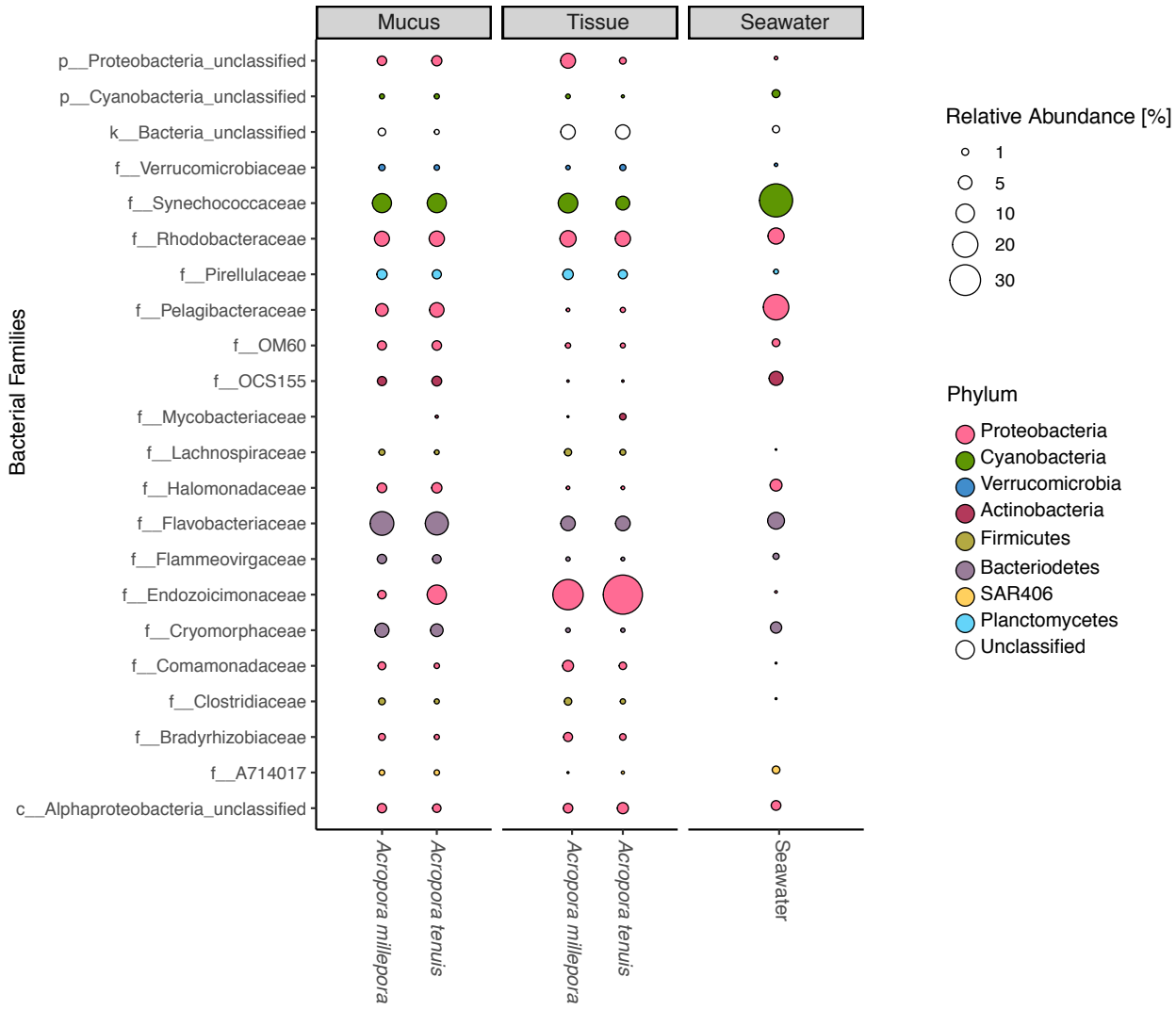

Figure 2.2 Microbial community composition (mean relative abundance) resolved for seawater and *Acropora* coral species (*A. tenuis* and *A. millepora*) for their surface mucus layer and tissue based on partial 16S rRNA amplicon sequencing. Only the 20 most abundant families across all samples are represented. Colours indicate the phylum-affiliation of the families.

Microbial community structure significantly differed among mucus, tissue and seawater (Figure 2.3; PERMANOVA, $F_{(2/223)}$= 21.75, p = 0.001), between *Acropora* species (PERMANOVA, $F_{(1/154)}$= 4.30, p = 0.001), season (PERMANOVA, $F_{(1/154)}$= 1.95, p = 0.002) and sampling location (PERMANOVA, $F_{(2/154)}$= 3.21, p= 0.001). Interaction between species and compartment was also significant (PERMANOVA, $F_{(1/154)}$= 2.95, p = 0.001) (other interactions can be seen in the Supplementary Table 1S).

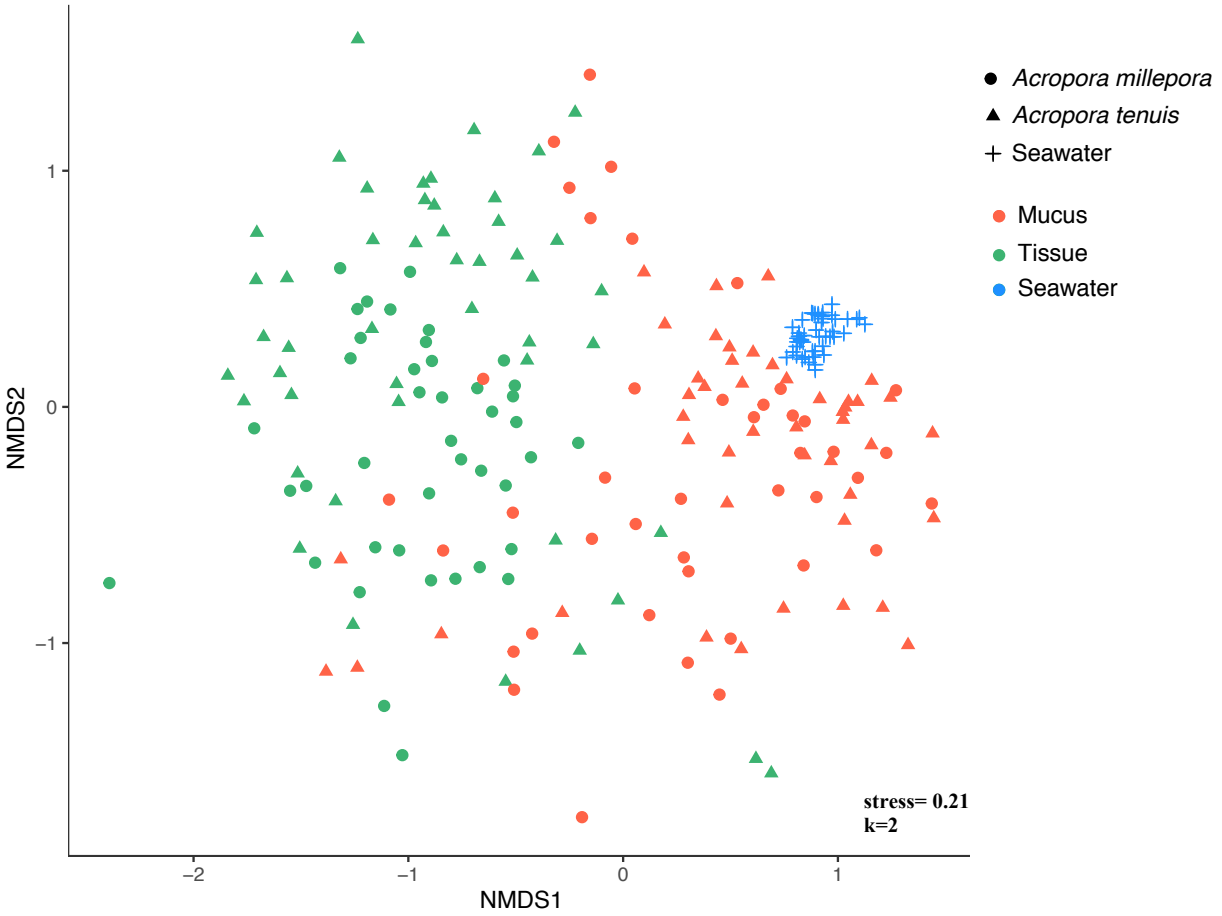

Figure 2.3 Two-dimensional non-metric multidimensional scaling (nMDS) ordination depicting variation in microbial community structure between coral compartments (mucus and tissue) of *Acropora tenuis and Acropora millepora*. Seawater samples were added as reference. "k" is the number of dimensions.

Physiological and environmental parameters

Physiological parameters measured (Chla/Proteins, Chla/Zooxanthellae, Zooxanthellae/Proteins) were tested for the statistical effect of coral species, sampling location and season. Results from three-way ANOVA showed no effect of species for any physiological factor, and season was significant only for Chla/Zooxanthellae (ANOVA, $F_{(1,78)} = 7.328$, p = 0.00834). Sampling location was a statistically significant factor for all the physiological parameters. A multiple comparison test (Tukey test) for sampling location demonstrated significant variation between samples originating from Magnetic Island (Geoffrey Bay) and the other two sites located at Orpheus Island (Pioneer Bay and Channel) (Table 2.3).

Table 2.3 Post hoc Tukey test for pairwise-comparisons of physiological variables across sampling locations. Significant *p-values* highlighted in bold.

Dependent variable	Comparison group	Compared with	Mean	*p* adj	CI (95%)	
					Lower	Upper
Chl*a*/Proteins	Geoffrey Bay	Channel	0.03	**0.004**	0.008	0.056
	Pioneer Bay	Channel	-0.01	0.434	-0.043	0.013
	Pioneer Bay	Geoffrey Bay	-0.04	**<0.001**	-0.071	-0.023
Chl*a*/Zoox	Geoffrey Bay	Channel	0.03	**0.016**	0.005	0.062
	Pioneer Bay	Channel	0.01	0.680	-0.022	0.046
	Pioneer Bay	Geoffrey Bay	-0.02	0.173	-0.050	0.006
Zoox/Proteins	Geoffrey Bay	Channel	0.05	0.089	-0.006	0.109
	Pioneer Bay	Channel	-0.06	0.059	-0.138	0.002
	Pioneer Bay	Geoffrey Bay	-0.11	**<0.001**	-0.177	0.061

* CI: Confidence interval.

Out of a total of 25 environmental variables, only 7 variables were included in the db-RDA analysis. The remaining variables were excluded due to numerous missing values in the metadata (Alkalinity (Alk), dissolved inorganic carbon (DIC), Alk/DIC) or due to collinearity. Selected variables were average raindays per month, salinity, concentration of particulate organic carbon (POC), total suspended solids (TSS), chlorophyll *a* (Chl*a*), ammonia (NH_4^+) and nitrite/nitrate (NO_2/NO_3^-). Table 2.4 shows the environmental variables selected for the analysis and their correlated variables.

Table 2.4 Matrix of correlated environmental variables with specific correlation values (Pearson's correlation). The variables on the top bar are the selected variables used for db-RDA. The variables on the left column are the excluded variables. Abbreviation of environmental variables as indicated: Total organic carbon in the sediment (TOC Sediment), Total organic nitrogen in the sediment (TON Sediment), Particulate organic carbon (POC), Particulate nitrogen (PN), Total nitrogen (TN), Non-purgeable organic carbon (NPOC), Non-purgeable inorganic carbon (NPIC), Phosphate (PO_4), Nitrogen dioxide (NO_2), Silica (SiO_2). Values only shown when module of Pearson's correlation ≥ 0.70.

Environmental Parameter	Average raindays	Salinity	POC	TSS	Chl*a*	NH_4^+	NO_2/NO_3^-
Average daylight							-0.82
Average temperature	0.95	0.71					
TOC Sediment	-0.70				0.76		
TON Sediment					0.82		
PN							0.83
TN		0.72			0.80		
NPOC				0.89	0.84		
NPIC		0.72			0.80		
PO_4	-0.72						
NO_2	-0.76						
SiO_2					0.73		
Grainsize percentage (2mm)							0.94
Grainsize percentage (<0.63µm)							0.95
Grainsize percentage (>0.63µm)							0.95

All environmental variables were affected by season and/or site, except salinity. While variation of average raindays, POC, NH_4^+ and NO_2/NO_3^- demonstrated effect of the interaction between season and site, TSS and Chl*a* showed only independent effects from one of the factors (Table 2.5). Summer samples were characterized by a higher number of raindays, higher Chl*a*,

higher NO_2/NO_3^-, higher POC, higher NH_4^+ and lower TSS as compared to winter samples (Figure S1).

Table 2.5 Two-way ANOVA results for season and site effects on non-collinear environmental variables measured from February 2016 to May 2017.

Factor	AVERAGE RAINDAYS		SALINITY		TSS		POC		Chl *a*		NH_4^+		NO_2/NO_3^-	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Season	103.77	**<0.001**	0.005	0.943	9.652	**<0.001**	0.105	0.747	0.637	0.430	18.753	**<0.001**	2.48	0.124
Site	11.12	**<0.001**	2.393	0.107	4.037	**0.026**	5.770	**0.006**	7.844	**0.001**	3.228	0.052	10.29	**<0.001**
Season x Site	17.71	**<0.001**	1.195	0.282	0.944	0.338	7.471	**0.009**	0.033	0.856	12.495	**0.001**	30.86	**<0.001**

**p* = *p*-value

Factors influencing host-associated and seawater microbiomes

The environmental/physiological parameters investigated in this study explained only a limited amount of the variation in the microbial community of mucus and tissue of the two *Acropora* species studied, as the first two db-RDA axes represented only a total of about 10-20% of variation (Figure 2.4). In contrast, the percentage of variation in seawater microbiomes explained by environmental variables was considerably higher, with the first two db-RDA axes accounting for a total of more than 40% of the variation (Supplementary Figure 1S).

Variation in seawater microbiome composition was mostly explained by environmental parameters, such as average raindays per month, NO_2/NO_3^-, NH_4^+, TSS, POC, and Chl*a* (Variation Partitioning Analysis, 49%), sampling location (Variation Partitioning Analysis, 16%) and season (Variation Partitioning Analysis, 7%) (ANOVA-like permutational test for Bray Curtis distance based Redundancy Analysis (dbRDA); Supplementary Table 2S). In total, these parameters explained 72% of the observed compositional variation in seawater microbiome. In comparison, for the mucus microbiome in *A. tenuis* and *A. millepora* environmental parameters only explained 15% and 12%, and sampling location 10% and 8%,

respectively, of the compositional variability, (ANOVA-like permutational test for dbRDA; Supplementary Table 2S). TSS and NH_4^+ were the common environmental variables that contributed as explanatory variables to the mucus microbiome in both *Acropora* species (total of 9% in *A. tenuis* and 7% in *A. millepora*). Host physiology (Chl*a*/zoox) also explained a small part of the variation in mucus microbiome of *A. tenuis* (3% of the observed variability). In total, the parameters analyzed for the *Acropora* mucus microbiome explained 28% (*A. tenuis*) and 20% (*A. millepora*) of the observed compositional variation. In contrast, the compositional variation in tissue differed more than the mucus microbiome between *Acropora* species. In *A. tenuis*, environmental parameters (TSS, POC and NO_2/NO_3^-), sampling location and physiological variables (Chl*a*/Proteins and Chl*a*/Zoox) were the factors that explained 15%, 14% and 7%, respectively, of the variation in the tissue microbiome (ANOVA-like permutational test for dbRDA; Supplementary Table 2S). Sampling location was the only factor that contributed for the compositional variation (Variation Partitioning Analysis, 10%) in the tissue microbiome of *A. millepora*.

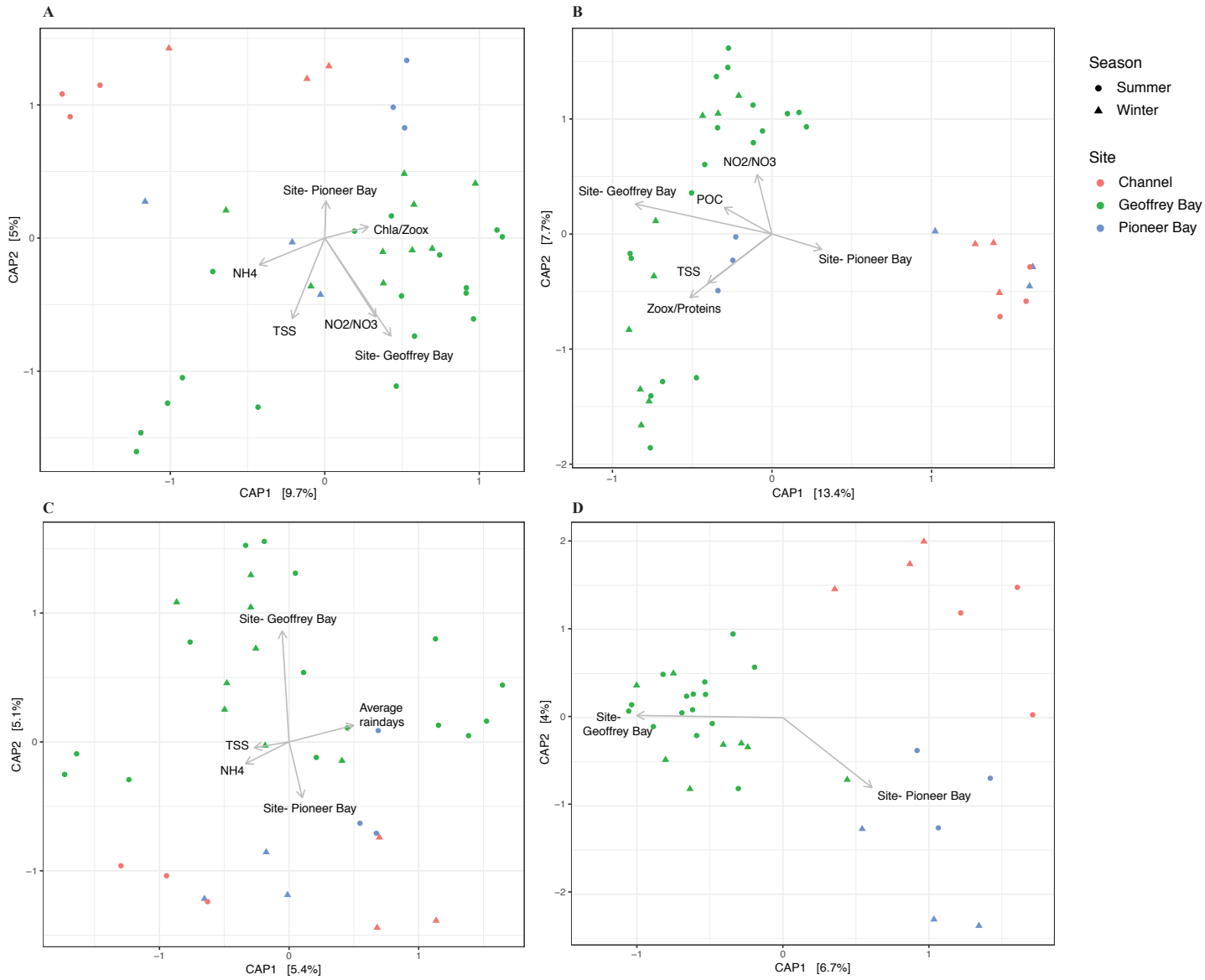

Figure 2.4 Distance-based redundancy analysis (db-RDA) of the relationship between environmental/physiological variables and the relative abundance of bacteria in (A) mucus microbiome of *Acropora tenuis,* (B) tissue microbiome of *Acropora tenuis*, (C) mucus microbiome of *Acopora millepora* and (D) tissue microbiome of *Acropora millepora*. Arrow length indicates the strength of the correlation between the variables and the samples. The two axes in plot A explained ca. 15% variance, in plot B explained ca. 20%, in plot C explained ca. 10% and in plot D explained ca. 10%.

Correlation between bacterial families and environmental/physiological parameters

A (Pearson's) correlation matrix illustrates the relationship between the relative abundance of the most abundant microbial families and environmental/physiological variables across different coral compartments of *Acropora* species (Supplementary Figure 2S and 3S). The results showed, for both compartments, a strong positive correlation between the relative abundance of copiotrophic families such as Rhodobacteraceae and Cryomorphaceae, and environmental factors as NH_4^+ and TSS. In contrast, an oligotrophic group of free-living *Alphaproteobacteria,* Pelagibacteraceae, present also in the mucus microbiome, was found to be negatively correlated with TSS and NH_4^+. Moreover, the family Pirellulaceae present in the mucus was positively correlated with NH_4^+ and NO_2/NO_3^-, while Halomonadaceae was negatively correlated with average raindays and NO_2/NO_3^- in *A. tenuis,* and negatively correlated with salinity in *A. millepora*. On the other hand, tissue-associated bacterial families showed a stronger significant correlation with TSS present in the surrounding environment. For example, families from the phylum Bacteroidetes, Cryomorphaceae and Flavobacteriaceae, were positively correlated with environmental TSS.

DISCUSSION

Microbial communities associated to corals are under constant influence of fluctuations in the environment and the physiology of their host. Previous studies have demonstrated changes in the coral microbiome under thermal stress (Ainsworth and Hoegh-Guldberg, 2009; Thurber et al. 2009; Lee et al. 2015; Grottoli et al. 2018), ocean acidification (Thurber et al. 2009; Grotolli et al. 2018), organic matter enrichment (Garren and Azam, 2012), bleaching events (Bourne et al. 2008) and other environmental and physiological factors (Guppy and Bythell, 2006; Li et al. 2014; Kelly et al. 2014; Pollock et al. 2018). Understanding the drivers of microbiome variation within a coral holobiont is crucial to better predict the effects of

environmental pressures on corals. However, much remains to be understood about the interactions established between corals and their symbionts. This study confirmed my hypothesis that environmental factors were the primary drivers of the mucus microbiome variation in both *Acropora* species. In contrast, the tissue microbiome differed between host species in its response to environmental and physiological factors, suggesting host-specific modulation of the environmental drivers of the tissue microbiome. Taken together, my results suggest that mucus microbiomes strongly respond to the surrounding environment, while tissue microbiomes show strong host species-specific control in their response to host physiology and environmental variation.

Microbial communities in coral compartments and seawater environment

This study confirms the differentiation of bacterial communities among mucus, tissue and surrounding seawater environment shown by previous studies (Bourne and Munn 2005; Sweet et al. 2011; Apprill et al. 2016; Engelen et al. 2018; Pollock et al. 2018). The higher similarity between mucus and seawater microbiomes (e.g. in alpha diversity, Table 2.2; and microbial composition) as compared to the similarity between tissue and seawater microbiomes in the present study suggests that the mucus receives more influence from the seawater environment than the tissue microbiome. Similar results were previously reported for other coral species (*Orbicella faveolata*, *Diploria strigosa*, *Montastraea cavernosa*, *Porites porites* and *Porites astreoides*), where mucus and seawater shared significantly more microbial taxa than those shared by tissue and seawater microbiomes (Apprill et al. 2016). Possible delivery sources of bacteria to the SML have been hypothesized. One of them is the passive settlement of bacteria from the external environment to the SML (Guppy and Bythell 2006; Sweet et al. 2011). For example, in the present study *Synechococcus* sp., a pelagic bacterium commonly found in seawater samples (Glasl et al. 2019b), was found in large abundances in the mucus

microbiome of *Acropora*. In general, the results showed significant differences in the microbial community composition between coral compartments (mucus and tissue) and also between closely related coral species (*A. tenuis* and *A. millepora*), hence confirming that coral microbiomes are host species- as well as microhabitat-specific.

Despite the host species-specificity of coral microbiomes, some bacterial taxa are ubiquitously associated with a particular coral compartment irrespective of host species. For example, the SML was dominated by Flavobacteriaceae and Synechococcaceae, while Endozoicimonaceae dominated the tissue microbiome in both coral species. However, microbiomes associated with distinct microhabitats of a coral also showed some overlap in their microbial community composition. Similar overlaps of SML and tissue microbiomes have previously been reported in other coral species (Sweet et al. 2011; Engelen et al. 2018; Weiler et al. 2018). This overlap is a natural feature of the coral holobiont as both compartments are within the same host and because the constituents of the SML are originally produced inside the tissue (Bythell and Wild, 2011). However, an overlap between SML and tissue microbiomes can also arise due to current limitations to retrieve samples exclusively originating from the mucus or tissue of a coral (Sweet et al. 2011; Weiler et al. 2018). Similar limitations could contribute to obscure differences between the mucus and seawater microbiomes, as these are in direct contact and SML is a rather hydrated environment (Brown and Bythell, 2005).

Drivers of mucus microbiome variation

We hypothesized that the mucus microbiome would be relatively more affected by environmental parameters, as the SML is in direct contact with the water column environment, whereas the tissue would be more affected by the physiology of the host. As expected, environmental parameters rather than host physiology appeared as the most influential factor significantly contributing to the variation of the mucus microbiome in both species (*A.*

millepora and *A. tenuis*). This agrees with recent studies relating changes in the mucus microbiome with environmental perturbations (Li et al. 2014; Pollock et al. 2018). However, the amount of influence from environmental parameters on the mucus (explaining 12-15% of variation) is relatively low as compared with the influence of those parameters on the seawater microbiome (explaining 49% of variation). This suggests that other relevant factors are possibly modulating the mucus microbiome. For instance, in *A. tenuis*, physiological parameters of the host also demonstrated some influence on the mucus microbiome (explaining 3% of variation). This indicates that variation in the surrounding environment and changes in the host physiology together can alter the bacterial community structure of the mucus. Mucus is a nutrient-rich medium fuelled by the photosynthetic activity of the zooxanthellae (Brown and Byhtell, 2005) and therefore it is expected that there is some degree of variation in its chemical composition that is explained by host-*Symbiodiniaceae* factors. Previously, links between mucus chemical composition and microbiome community structure have been proposed (Tremblay et al. 2011). However, mucus is highly hydrated: mucocyte cells originally release their secretions in a condensed form, which then undergoes a massive swelling on hydration, to finally form a visco-elastic gel (Brown and Byhtell, 2005). This process entails that SML can be influenced by the presence of nutrients dissolved in the surrounding seawater.

NH4, TSS, POC, average raindays per month and NO2/NO3 were environmental factors found by the present study to significantly influence the mucus microbiome in *Acropora* species. Li et al. (2014) and Chen et al. (2011) also suggested that rainfall had a crucial effect on bacterial community variation in the coral microbiome, being mostly associated with an increase in the relative abundance of the *Bacilli* group (Li et al. 2014; Chen et al. 2011). However, in the present study, NH4 and TSS seemed to be the most influential variables out of the measured ones, as they were significant for both species (Figure 2.3). The coral holobiont is very efficient in taking up nitrogen, as nitrogen is required by the photosynthesis production

of their zooxanthellae symbionts (Rädecker et al. 2015). Ammonia is taken up by members of the mucus microbiome such as nitrifying Archaea (Siboni et al. 2008), therefore making nitrate and nitrite available for assimilation by the coral holobiont. Furthermore, corals exposed to suspended sediment can be affected in numerous ways. TSS can impact corals by limiting light availability for photosynthesis, decreasing zooxanthellae densities, smothering of tissues and enhancing diseases on corals, which can indirectly affect microbial communities (Fabricius, 2005; Pollock et al. 2014). Increased sedimentation also increases mucus production or sheeting to remove sediment (Brown and Bythell, 2005; Klaus et al. 2007).

Rhodobacteraceae and Cryomorphaceae, two copiotrophic representatives, showed a positive correlation with increasing TSS and NH4 (Figure 2S and 3S), while the alphaproteobacterial family Pelagibacteraceae, mostly comprised of oligotrophic bacteria, showed negative correlation with TSS and NH4 (Figure 2S and 3S). In a previous study, the abundance of Rhodobacteraceae was also strongly correlated with salinity, oxygen saturation, pH, and nitrate concentration in seawater samples (Campbell et al. 2015). Oligotrophic organisms are adapted to environments that offer very low levels of nutrients and copiotrophic organisms are usually present in nutrient-rich waters (Poindexter, 1981). Therefore, the abundance of oligotrophic and copiotrophic bacterial families are expected to increase or decrease based on the nutrient availability in the environment. Based on this, I postulate that the relative abundance of different functional groups in the mucus microbiome could inform about changes in environment conditions.

Drivers of tissue microbiome variation

In contrast, the response of the coral tissue microbiome to environmental and physiological parameters greatly differed between species. Only the tissue microbiome from *A. tenuis* responded to environmental and physiological parameters analysed, while *A. millepora*

did not respond to any parameter except sampling location. This result obtained for closely related species suggests that coral species, in general, differ in the response of their tissue microbiome to environmental and physiological parameters. The tissue microbiome of *A. millepora* likely hosts a more temporally stable community than *A. tenuis*, hence, meaning the microbiome is less sensitive to physiological and environmental variation. Since both species host a similar microbiome diversity (Table 1), the abovementioned difference can be associated to specific features of each species, through which *A. millepora* could modulate the internal environment to creating more stable intra-tissue conditions than *A. tenuis*. A possible explanation for this is the influence of the algal symbiont (Symbiodiniaceae) type associated to the host. Little et al. (2004) investigated Symbiodiniaceae communities associated with *A. millepora* and *A. tenuis* on Magnetic Island. Their study demonstrated that the coral-algal endosymbiotic relationship in *Acropora* spp. is both dynamic and flexible (corals associate with different Symbiodinaceae type at different life stages, for example) and contributes significantly to physiological attributes of the coral holobiont. *Acropora* corals may select the most appropriate symbiont at a specific stage of their life to meet their energy requirements (Little et al. 2004). Also, environmental factors such as thermal stress can lead to temporal changes in the symbiont community and this can vary specifically or individually (Cooper et al. 2011; Howells et al. 2011; Rocker et al. 2012). As the microbiome is strongly associated to the coral holobiont, any disturbance in the host-endosymbiotic relationship may have indirect effects on the microbial composition and their response to environmental and physiological factors. Other studies demonstrate the influence of Symbiodinaceae on the host microbial community and also support the idea that these two components of the coral holobiont are finely tuned (Littman et al. 2009; Littman et al. 2010; Glasl et al. 2017a; Grottoli et al. 2018).

In regard to microbial composition in the tissue, the families Cryomorphaceae and Flavobacteriaceae were significantly positively correlated with TSS concentration. Increased

TSS concentrations in the seawater are considered a limiting factor for photosynthetic activity and, consequently, coral growth (Parwati et al. 2014). Furthermore, TSS was found to positively correlate with reduced coral reef cover area and the occurrence of coral diseases (Parwati et al. 2014; Gignoux-Wolfsohn et al. 2017). Furthermore, planktonic Cryomorphaceae and Flavobacteriaceae were recently described as putative microbial indicators of coral reef environmental perturbations (Glasl et al. 2019a). Hence, the increase of Cryomorphaceae and Flavobacteriaceae in the coral tissue may provide a putative indicator of coral holobiont health in relation to turbidity.

Spatial and temporal influence on the coral microbiome

Although many studies have focused on the spatial structure and temporal stability of coral-associated bacteria, their conclusions are often contradictory. For example, whilst some studies showed bacterial communities from the same coral species to exhibit a consistent community structure across space and time (Rowher et al. 2002), others reported on seasonal and spatial variation (Littman et al. 2009; Ceh et al. 2011; Chen et al. 2011; Kimes et al. 2013). In the present study, both spatial and temporal microbiome variation were identified, superimposed on specificity imposed by host species and compartment. When temporal effects were broken down to the individual contribution of a number of environmental parameters, seasonal variation did not significantly explain any further variation in the mucus or tissue microbiome of *Acropora* species (although it still explained 7% of the variation in seawater microbiome). This suggests that seasonal fluctuation was well captured by the environmental parameters measured in this study. On the contrary, sampling location was a crucial factor showing significant influence on bacterial community variation of both coral compartments, even when individual environmental parameters were tested in our dbRDA models.

In order to disentangle the effects of time and space (which together shape environmental variation), a specific analysis performed only on the dataset from Geoffrey Bay (the one with the highest temporal resolution) resulted in fewer significant variables explaining the variation on the mucus and tissue microbiomes for both *Acropora* species (Supplementary Table 3S), as compared to the main analysis (including all sampling sites). This suggests that some variables selected in the main analysis (e.g. POC) represented spatial variation and not temporal variation exclusively. Furthermore, there was a clear separation between samples from different islands; i.e., differences between sampling locations were more emphasized between Magnetic island and the two sampling sites on Orpheus island (as shown in Figure 2.3). In the past, differences in coral microbial community between Magnetic and Orpheus islands were also shown by Littman et al. (2009). Their results indicate that certain bacterial groups present in the tissue microbiome are specifically associated with the genus *Acropora*, but the dominant bacterial genera differed between islands. This might be associated with distinct abiotic conditions of each island. For example, reefs surrounding Magnetic Island are more exposed to anthropogenic impacts as compared to reefs on Orpheus Island (Muslim and Jones, 2003). Enrichment of nutrients, low water quality, turbidity, and other stressors related to anthropogenic disturbance can strongly affect microbial communities. However, the spatial influence on the microbial communities can also be related to the dominant type of Symbiodiniaceae associated with the coral host at each island. As demonstrated by Császár et al. (2010), *A. millepora* associates with two different Symbiodiniaceae genera across these specific GBR sites: the genus *Durusdinium* (thermo-tolerant) is dominant on Magnetic Island, while genus *Cladocopium* type C2 (intermediately tolerant) is more prevalent around Orpheus island. As mentioned above, environmental variation can alter the dominance of Symbiodiniaceae lineages associated to the host, hence, leading to a subsequent variation in the

microbial community. Future studies are needed to further ascertain the exact contribution of the spatial drivers of coral microbiomes.

CONCLUSION

In summary, this study highlights that microbiomes inhabiting different physical niches within the coral holobiont differ in their response to host and environmental factors. Microbiomes of *Acropora* spp. differed significantly among compartments (mucus and tissue) and species (*A. tenuis* and *A. millepora*), and were also influenced by sampling location and season. Coral mucus from both species showed greater influence of environmental parameters as compared to that of host physiological parameters. In contrast, the tissue microbiomes of the two *Acropora* species showed different response to environmental/physiological parameters, suggesting host-specific modulation of the environmental drivers of the tissue microbiome. By comprehensively investigating spatial-temporal variation in environmental and physiological drivers of microbiome variation for distinct coral compartments in closely related species, this study contributes to disentangle the factors controlling microbiome composition in corals. This study helps to clarify the limited and controversial knowledge on coral microbiome and its dynamics, and also stimulates the use of more holistic methodological approaches on this topic. Further studies should include other coral species and evaluate the factors identified in the present study experimentally to provide a broader understanding of microbiome variation in corals.

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ANNEXES

SUPPLEMENTARY TABLES

Table 1S. Permutational multivariate analysis of variance (PERMANOVA) table for interactions among microbial communities from distinct coral species (A. tenuis and A. millepora), coral compartments (mucus and tissue), season (summer *versus* winter) and sampling site (Geoffrey Bay, Pioneer Bay and Channel).

Source of Variation Interactions	***df***	**Pseudo-*F***	***P*(perm)[1]**
Species:Season	1	1.0254	0.384
Compartment:Season	1	1.4994	**0.008**
Species:Site	2	2.0424	**0.001**
Compartment:Site	2	2.2557	**0.001**
Season:Site	2	1.9736	**0.001**
Species:Compartment:Season	1	0.9558	0.564
Species:Compartment:Site	2	1.9625	**0.001**
Species:Season:Site	2	1.0795	0.228
Compartment:Season:Site	2	1.6571	**0.001**
Species:Compartment:Season:Site	2	1.0449	0.296

[1]Significant results (p(perm) <0.05) are highlighted in bold

Table 2S. ANOVA-like permutational test for dbRDA table for significant environmental and physiological variables selected by model selection for each compartment/ species.

Source of Variation	Mucus *A. tenuis*		Tissue *A. tenuis*		Mucus *A. millepora*		Tissue *A. millepora*	
	Pseudo- *F*	P(perm)[1]	Pseudo- *F*	P(perm)[1]	Pseudo- *F*	P(perm)[1]	Pseudo- *F*	P(perm)[1]
Site	1.75	**0.002**	3.21	**0.001**	1.61	**0.001**	1.91	**0.001**
TSS	2.44	**0.001**	2.30	**0.003**	1.51	**0.001**		
NH4	1.74	**0.003**			1.75	**0.001**		
NO2/NO3	1.45	**0.026**	1.94	**0.007**				
POC			2.12	**0.005**				
Average raindays					1.69	**0.001**		
Zoox/Proteins			2.78	**0.001**				
Chl*a*/Zoox	1.36	**0.040**						

[1]Significant results (p(perm) <0.05) are highlighted in bold

Table 3S. Permutational multivariate analysis of variance (PERMANOVA) for Bray Curtis distance based Redundancy Analysis (dbRDA) table for significant environmental and physiological variables selected by model selection for each compartment/ species only for Geoffrey Bay.

Source of Variation	Mucus *A. tenuis*		Tissue *A. tenuis*		Mucus *A. millepora*		Tissue *A. millepora*	
	Pseudo- *F*	P(perm)[1]	Pseudo- *F*	P(perm)[1]	Pseudo- *F*	P(perm)[1]	Pseudo- *F*	P(perm)[1]
TSS	2.73	**0.001**	2.30	**0.003**				
NH4	1.69	**0.009**						
NO2/NO3			1.94	**0.007**			2.20	0.004
Chl *a*	2.55	0.002	2.12	**0.005**				
Salinity					2.15	**0.005**		
Zoox/Proteins			3.64	**0.001**				
Chl*a*/Proteins	1.40	**0.046**						

[1]Significant results (*p*(perm) <0.05) are highlighted in bold

SUPLEMENTARY FIGURES

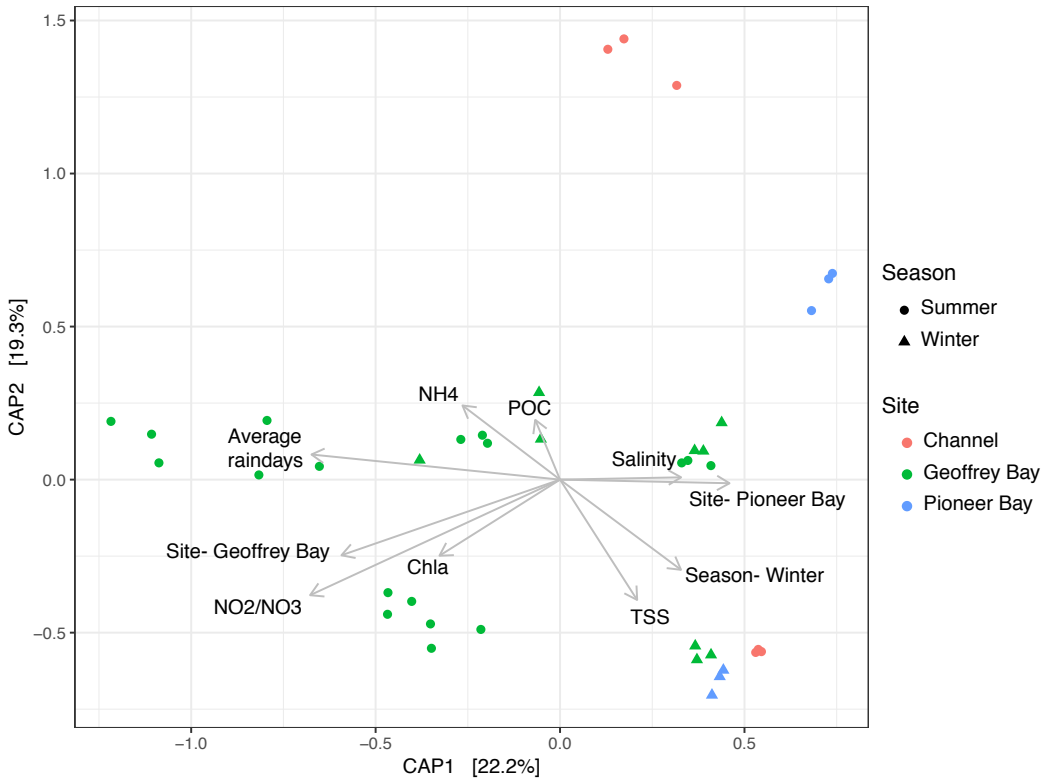

Figure 1S. Distance-based redundancy analysis (db-RDA) of the relationship between environmental variables and the relative abundance of seawater microbiome. Arrows indicate the strength of the correlation between the variables and the samples. The correlation is stronger as longer the arrow length. The two axes in the plot explained ca. 40% variance.

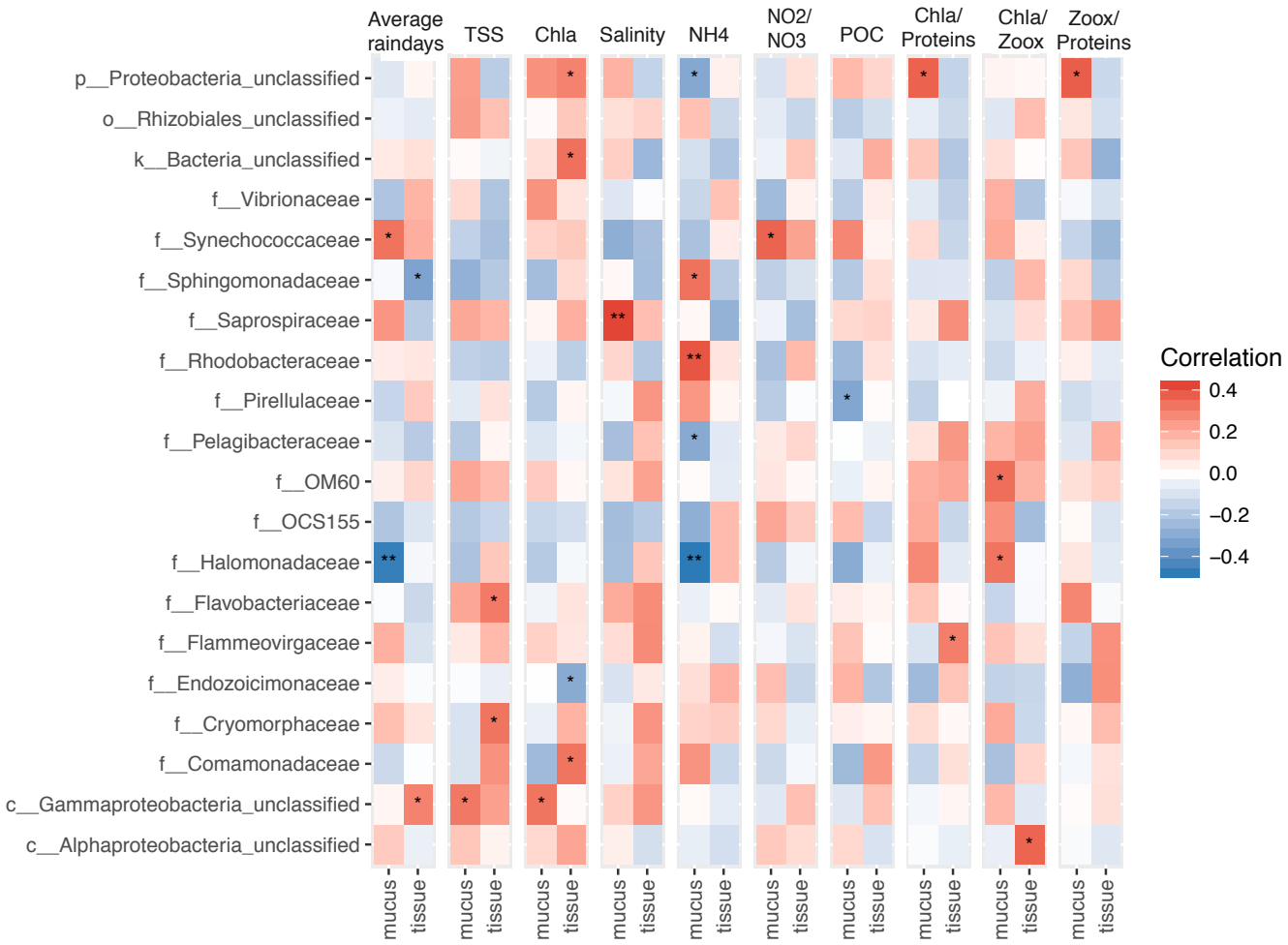

Figure 2S. Correlation matrix based on Pearson's correlation among the 10 most abundant bacterial families and environmental/ physiological variables in *Acropora tenuis.*

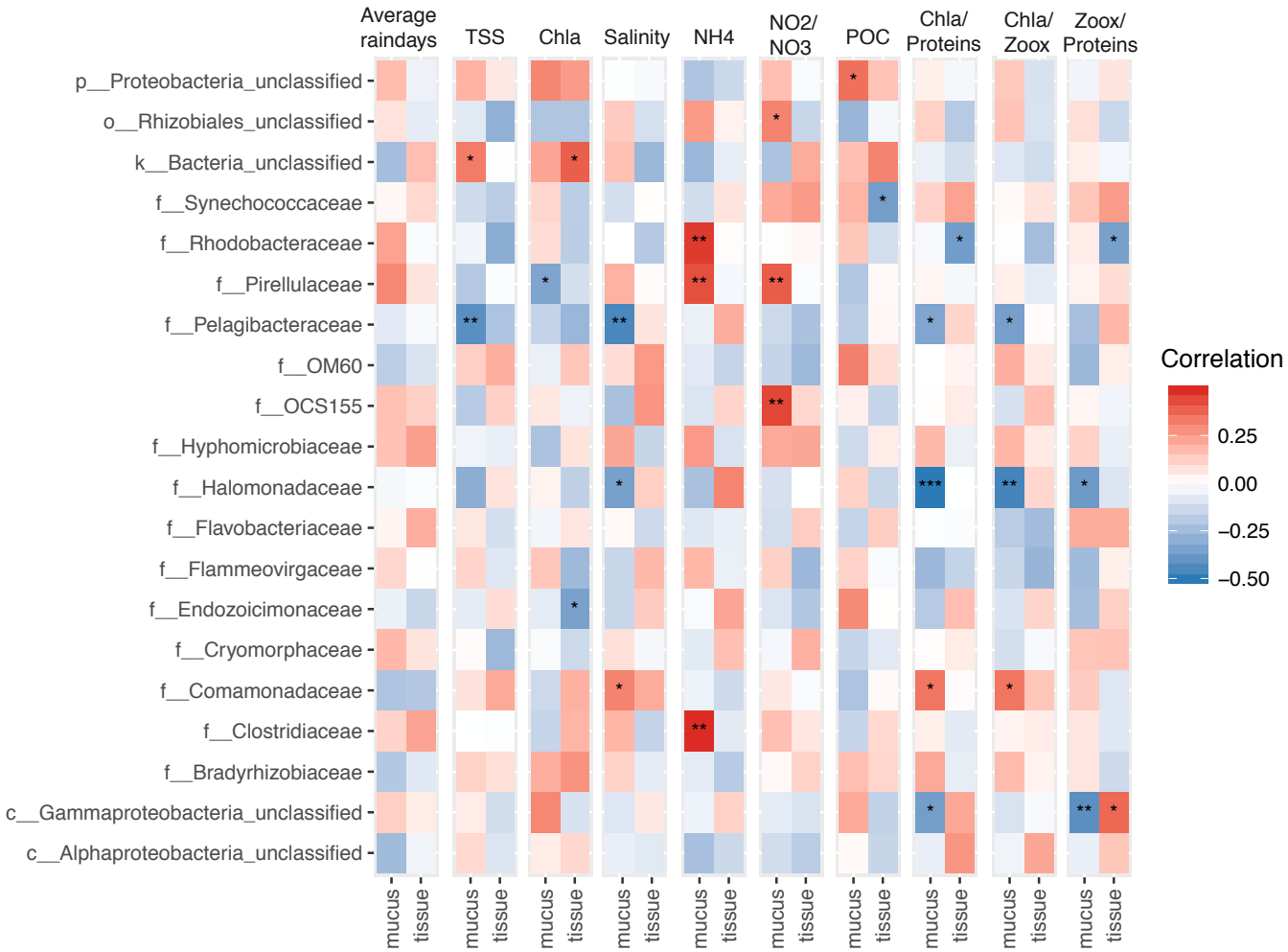

Figure 3S. Correlation matrix based on Pearson's correlation among the 10 most abundant bacterial families and environmental/ physiological variables in *Acropora millepora*.