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MICROBIAL INDICATORS FOR ENVIRONMENTAL STRESS AND ECOSYSTEM HEALTH ASSESSMENTS

submitted by

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for the degree of Doctor of Philosophy

at the College of Science and Engineering, James Cook University

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My PhD journey has brought me to one of the most fascinating places; a place where it is possible to live side-by-side with the oldest living culture on earth. I would like to express my deepest gratitude to the traditional owners, the Bindal, Wulgurukaba and Manbarra people, of the land and sea country where this research was conducted. I pay my greatest respects to the Elders past, present and future.

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AUTHORSHIP DECLARATION

Chapter No.	Details of publication(s) on which the chapter is based	Nature and extend of the intellectual input of each author, including the candidate	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis
1	Glasl B, NS Webster and DG Bourne (2017) Microbial indicators as a diagnostic tool for assessing water quality and climate stress in coral reef ecosystems. *Marine Biology* 164:91.	Glasl wrote the first draft of the manuscript which was revised with the editorial input from Webster and Bourne. Glasl developed the figures and tables.	Name: Nicole S. Webster Signature: Name: David G. Bourne Signature:
2	Glasl B, DG Bourne, PR Frade and NS Webster (2018) Establishing microbial baselines to identify indicators of coral reef health. *Microbiology Australia* 10.1071/MA18011.	Glasl wrote the first draft of the manuscript which was revised with the editorial input from Bourne, Frade and Webster. Glasl developed the figures and tables.	Name: Nicole S. Webster Signature: Name: David G. Bourne Signature:
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7	Glasl B, S Robbins, PR Frade, E Marangon, PW Laffy, DG Bourne and NS Webster (submitted to ISME J) Comparative genome-centric analysis reveals seasonal variation in the function of coral reef microbiomes	All authors contributed to the conception and design of the study. Samples were collected by Glasl, Bourne, Frade and Webster. Samples were processed in the laboratory by Glasl and Frade. Glasl analysed and prepared the manuscript (including figures and tables). All authors revised the final manuscript.	Name: Nicole S. Webster Signature: Name: David G. Bourne Signature:

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GENERAL ABSTRACT

The health of coral reef ecosystems is declining globally due to the cumulative effects of local pressures such as eutrophication and over-fishing as well as global pressures such as rising sea surface temperatures that result in mass coral bleaching events. Early identification of adverse environmental conditions and declining ecosystem health is important to effectively implement management strategies that can mitigate the effects of environmental pressures on threatened coral reef ecosystems. Microorganisms, as fundamental members of coral reef ecosystems, contribute to the future trajectories of coral reefs, however despite their pivotal roles in coral reef ecosystem functioning and stability, the potential value of microorganisms for monitoring ecosystem health remains largely unexplored. To comprehensively assess the utility of microorganisms as a diagnostic tool for monitoring coral reef health, this thesis provides outcomes from an extensive literature review, large-scale field collections, aquaria-based experimental procedures, and in depth sequencing analyses. In Chapters 1 and 2, I elaborate in detail on the implementation of biological indicator approaches, the potential role of microbial indicators in coral reef monitoring programs, the function and diversity of host-associated (i.e., coral, sponge and macroalgae) and free-living (i.e., seawater and sediment) microbiomes, and on state-of-the-art methods that can be used to identify microbial indicators. One of the main hurdles to implement microbial monitoring into current monitoring initiatives for the Great Barrier Reef (GBR) is the lack of microbial reference datasets combined with the high complexity and diversity of coral reef microbial communities. Therefore, this PhD thesis aims to establish the first taxonomic and functional microbial reference datasets for the GBR using state-of-the-art meta-omic sequencing techniques (Chapter 3), and to identify the most suitable reef microbiomes for a microbial indicator program to pinpoint environmental state (Chapter 4-7).

To achieve the main aim of my thesis, I successfully established the first comprehensive taxonomic and functional microbial baseline for selected GBR sites. Microbial samples for meta-omic sequencing were collected monthly over a 16-month period from multiple host-associated (e.g., coral, sponge and macroalgae) and free-living (e.g., seawater and sediment) microbial habitats (Chapter 3, 4 and 7). In total, 381 samples were analysed by amplicon-based 16S rRNA gene sequencing, 42 samples by metagenomic sequencing and 36 samples by metatranscriptomic sequencing. To quantify the diagnostic potential of host-associated and free-living microbiomes, I applied a series of multivariate statistical approaches to the microbial reference dataset based on community composition (Chapter 4). The seawater microbiome was identified as having the greatest diagnostic potential to infer shifts in the

surrounding reef environment due to its high habitat specificity, its uniform community response pattern, and its environmental sensitivity. The high diagnostic value of the seawater microbiome was further confirmed by applying a suite of indicator value and machine learning approaches, which provided an accurate prediction of temperature and eutrophication state (i.e., chlorophyll concentration and turbidity) based on a given microbial community.

Through a set of aquarium-based experiments, I experimentally validated the high stability of host-associated microbiomes (i.e., sponge and coral) to non-lethal environmental disturbances (i.e. low salinity stress exposure under current and future reef scenarios; Chapter 5 and 6). The fundamental aim of these experiments was to explore the effect of low *versus* high microbiome diversity on sponge holobiont stability (Chapter 5) and to disentangle the effect of host-genotype and environment on coral-associated microbiomes (Chapter 6). In both experiments, changes in the microbial communities were evaluated over time using 16S rRNA gene sequencing. The sponges and corals exhibited no signs of stress and their microbiomes remained highly stable, irrespective of the diversity of the microbiome (Chapter 5) and the degree of stress applied (Chapter 6). However, microbiome composition in both corals and sponges varied significantly between host individuals (genotypes), which further confirms the limited utility of host-associated microbiomes as sensitive markers for environmental disturbances.

In addition to the extensive characterisation of compositional variation in host-associated and free-living microbiomes, I aimed to identify the functional role of individual members of coral reef microbiomes using a genome-centric metagenome approach (Chapter 7). The functional potential of individual microbial taxa was assessed in relation to seasonal macroalgal abundance and sea-surface temperature fluctuations. In total, 125 high quality metagenome-assembled genomes (MAGs) were reconstructed, belonging to 15 bacterial and 3 archaeal phyla. I showed that changes in the abundance of individual microbial taxa due to the cumulative effects of macroalgae proliferation and increasing sea-surface temperature have implications for the functional potential of the microbiome and hence the stability of benthic holobionts and the reef ecosystem. For example, the increase in macroalgae and sea-surface temperature 1) correlated with an increased potential for algal-derived polysaccharide (e.g. laminarin) degradation by planktonic Bacteroidota, 2) resulted in an increase in the Firmicutes to Bacteroidota ratio in macroalgae biofilms which has implications for biofilm succession, polysaccharide degradation and antibiotic production, and 3) led to an 85% decrease in the relative abundance of the symbiont phylum Chloroflexota in the sponge tissue, potentially affecting nutrition, waste product removal and detoxification of the sponge holobiont.

In summary, this thesis provides a framework for the integration of microbial based monitoring approaches into current coral reef monitoring initiatives and highlights differences in environmental sensitivity between host-associated and free-living microbiomes. Furthermore, this thesis establishes the first holistic microbial baseline for selected GBR sites, identifies the seawater microbiome as the most suitable reef microbiome for a microbial indicator program, and elucidates the functional potential of individual microbial members of host- and free-living microbiomes in a coral reef ecosystem.

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Chapter 1

GENERAL INTRODUCTION: MICROBIAL INDICATORS AS A DIAGNOSTIC TOOL FOR ASSESSING WATER QUALITY AND CLIMATE STRESS IN CORAL REEF ECOSYSTEMS

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Abstract

Microorganisms play a fundamental role in the functioning and stability of coral reef ecosystems. However, environmental disturbances can trigger alterations to the natural microbial community composition and their functional traits with potentially detrimental consequences for host organisms, such as corals, sponges and algae and concomitant implications for the entire coral reef ecosystem. Coral reefs are increasingly affected by localized impacts such as declining water quality and global pressures derived from human-induced climate change, which severely alters the natural conditions on reefs and can push dominating benthic life forms towards the limit of their resistance and resilience. Microorganisms can respond very rapidly to these altered environmental conditions so defining their natural variability over spatial and temporal gradients is critical for early and accurate identification of environmental disturbances. The rapid response of microbes to environmental change is likely to confer significant advantages over traditional reef monitoring methods, which are based on visual signs of health deterioration in benthic coral reef macroorganisms. This review discusses the potential of microbes as early warning indicators for environmental stress and coral reef health and proposes priorities for future research.

Introduction

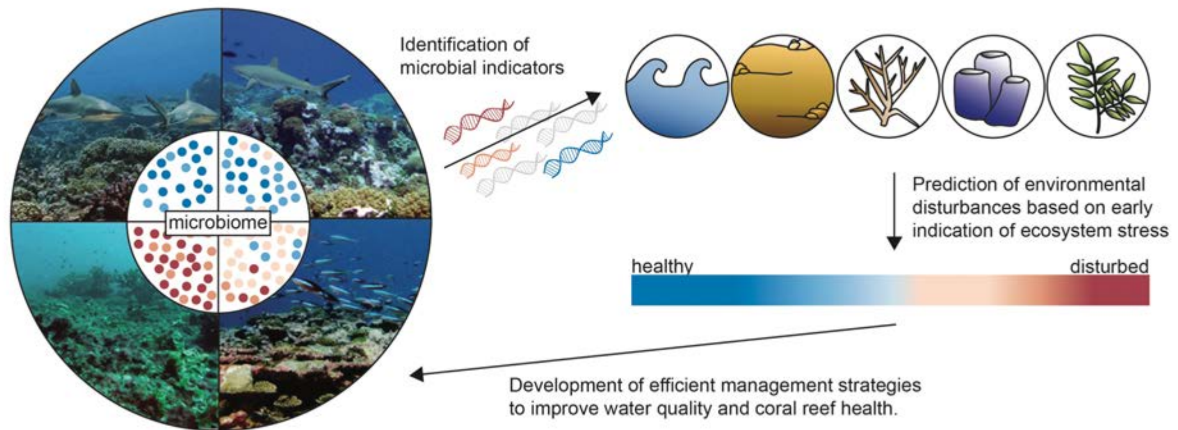
Coral reefs have flourished in tropical shallow water regions for over 240 million years (Stanley and Fautin 2001). However, during the last 30 years, coral reefs have faced severe threats due to anthropogenic climate change, crown of thorns starfish (COTS), disease, overfishing and pollution (De'ath et al 2012, Hoegh-Guldberg 2011). For instance, the emission of carbon dioxide (CO_2) leads to a decrease in the ocean's pH and adversely affects calcium carbonate deposition by calcifying organisms such as environmentally susceptible coral species (Hoegh-Guldberg et al 2007, Putnam et al 2016). Increased ocean surface temperature as a consequence of global warming, not only results in more frequent and severe tropical storm events (Knutson et al 2010) but also pushes the coral-Symbiodiniaceae symbiosis towards its thermal tolerance limit (Hoegh-Guldberg 1999). Once the temperature exceeds the resistance threshold of the symbiosis, the interaction between corals and their photoautotrophic Symbiodiniaceae breaks down and corals bleach (Brown 1997). Mass coral bleaching events have occurred more frequently over the last decades (Baker et al 2008, Donner et al 2005, Hoegh-Guldberg et al 2007). The most recent global bleaching episode in 2015-2016 was the longest bleaching event recorded in history (U.S. National Oceanic and Atmospheric Administration) and has led to severe bleaching worldwide. In the northern sector of the Great

Barrier Reef (GBR) in Australia, this bleaching event caused the mortality of up 29% of all corals (Great Barrier Reef Marine Park Authority 2017, Hughes et al 2018a). In addition to the hazards emerging from global change, localized reef-scale anthropogenic impact can further influence the ecological resilience of corals and other reef organisms to global stressors (Knowlton and Jackson 2008). For example, overfishing has led to an alteration of the trophic food web (Valentine and Heck 2005). With the removal of herbivorous fish from coral reefs, macroalgae and turf algae are less efficiently grazed. This can result in a phase shift from coral to macroalgae dominated reefs (Burkepile and Hay 2006, Vermeij et al 2010). Changes in land-based practices can also impact reef ecosystems with land-clearing, coastal urbanization and intense agricultural practices contributing to decreased water quality on reefs, which in turn further contributes to declining reef health (De'ath and Fabricius 2010).

Monitoring programs, such as ‘Reef Check’ and ‘The Global Coral Monitoring Network’ use visual surveys of reef associated fish, invertebrates, substrate composition, reef damage and disease impacts to assess reef health (Hill and Wilkinson 2004, Hodgson 2001). Most visual cues (e.g., tissue necrosis, mortality and shifts in community composition) only become evident in the advanced stages of coral reef ecosystem stress, when ecosystem health and resistance are already compromised. At this stage, the ability to recover is dependent on the resilience of the ecosystem and the success of management interventions is jeopardized. Hence, the development of a reliable early warning system that facilitates management intervention before severe damage occurs is clearly warranted.

Microorganisms are key drivers of large-scale biogeochemical cycles in the oceans (Azam et al 1983, Falkowski et al 2008, Whitman et al 1998) and also play a fundamental role in coral reef ecosystem functioning, through mediating nutrient cycling (Charpy et al 2012, Tout et al 2014). Furthermore, microbes live in intimate relationships with benthos-dominating life forms such as corals, sponges and macroalgae, where they have a vital role in host fitness through additional nutrient provision, removal of waste products and the exclusion of opportunistic microbial pathogens (Blackall et al 2015, Bourne et al 2016, Egan et al 2013, Webster and Thomas 2016). However, disturbance events can alter the natural microbial community structure, abundance and metabolic functions either directly or indirectly (Shade et al 2012). Disturbance-related deviations from the naturally occurring microbial communities may provide useful indications for coral reef ecosystem stability and facilitate sensitive predictions of environmental stress (Figure 1.1).

This review aims to assess the utility of microorganisms as a diagnostic tool for assessing water quality and climate-driven stress in the coral reef ecosystem. Insights are provided into (1) the implementation of biological indicator approaches; (2) the potential role of microbial indicators in coral reef monitoring programs; (3) the function and diversity of



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microbes associated with dominating benthic life forms (corals, sponges and macroalgae) and their surrounding environments (seawater and sediment) and (4) how state-of-the-art methods used to study microbial community composition and function can be implemented into diagnostic tools to assess coral reef ecosystem health.

Biological indicators: definition, function and application

Biological indicators, also referred to as indicator species, are organisms that can be used to monitor habitat conditions and environmental changes based on their niche preferences (De Cáceres et al 2010, McGeoch and Chown 1998). The presence/absence or abundance values of selected organisms or communities at specific sampling sites are monitored, allowing conclusions to be drawn on the ecological integrity of a certain ecosystem (Carignan and Villard 2002). Depending on the response time, bioindicators can either be used to detect severe short-term stress events or to better understand the long-term effect of chronic stress exposure (Cairns and Pratt 1993). The use of taxa with moderate tolerance towards environmental variability is favoured against rare or ubiquitous taxa, which respectively show high sensitivity or high tolerance towards environmental perturbations (Holt and Miller 2011). Cooper et al (2009) identified the following five selection criteria, to be considered when choosing bioindicators: specificity, monotonicity, natural variability, practicality and ecological relevance (see Table 1.1).

Table 1.1. Summary of criteria for the selection of microbial indicators to monitor water quality changes and predict stressors in coral reef ecosystems. Criteria were taken from Cooper et al. (2009) and definitions were adapted to fit the microbial indicator approach. Selected examples of studies examining microbial community responses from marine systems are provided to support how this indicator approach can satisfy the identified criteria.

Criteria	Definition	Examples	Source
Specificity	Compositional and/or functional shifts reflect specific responses to environmental stressor	Coral microbiome shifts towards an opportunistic bacterial community and shows an increase in the abundance of virulence genes in response to bleaching, environmental stressors and anthropogenic impact.	Bourne et al 2008; Dinsdale et al 2008; Vega Thurber et al 2009; Littman et al 2011; Kelly et al 2014
		Heavy metal pollution, increased temperature and elevated pCO_2 can lead to changes in the composition and function of the sponge microbiome.	Webster et al 2001; Webster et al 2008; Selvin et al 2009; Fan et al 2013; Morrow et al 2015
Monotonicity	The magnitude of microbiome disruption reflects the intensity and duration of the stressor	The resistance and resilience of the microbial community composition and functions varies between short and long-term disturbances	reviewed by Shade et al 2012
Variability	Natural variability and dynamics of the microbiome forms the baseline of a healthy microbial community. Deviations to the baseline can be used as indicators for changes in the ecosystem.	Planktonic community composition and function varies between different coral reef niches and along a gradient of water quality.	Tout et al 2014; Angly et al 2016
		The coral microbiome is described as host species specific, however, short and long-term temporal variation have been observed.	Rohwer et al 2002; Li et al 2014; Glasl et al 2016
		Sponge-associated microbes form a stable symbiotic relation with the host over time and seasons.	Erwin et al 2012; Thomas et al 2016
Practicality	Prokaryotic indicator analysis should be cost and time efficient, easy to measure, non-destructive and observer independent.	Technological advances allow cost and time efficient analysis of prokaryotic communities *in situ* (e.g., next generation sequencing techniques, meta-omic approaches)	Schuster 2008
Relevance	Prokaryotic shifts should be ecologically relevant and enable prediction of environmental stress and hence, ecosystem health.	Prokaryotes have fundamental roles in coral reef functioning but also in coral and sponge holobiont health.	Bourne and Webster 2013

Kolkwitz and Marsson (1908) developed one of the first indicator approaches to evaluate the degree of pollution in rivers based on the occurrence of saprobic micro- (e.g., ciliates and flagellates) and macroorganisms (e.g., insect larvae, molluscs, bivalves, annelids and crustaceans). Since then, various biomonitoring programs have been developed and successfully applied in estuarine and freshwater ecosystems by assessing indicator species within the macrobenthic invertebrate community such as the AUSRIVAS - Australian River Assessment Scheme (Smith et al 1999), RIVPACS - River Invertebrate Prediction and Classification System (Wright 1995), SIGNAL - Stream Invertebrate Grade Number Average Level (Chessman 1995), and WFD - European Union Water Framework Directive (Parliament 2000). Biomonitoring approaches also find application in coral reef health monitoring (Cooper et al 2009). For example, the FORAM index (Hallock et al 2003) provides insights into the water quality of coral reefs by quantifying size classes of Foraminifera in the upper sediment layer.

Although indicator approaches are frequently used in conservation biology, land management, landscape mapping and in the design of protected areas, standardized approaches and statistical methods to identify suitable indicators are still scarce. Dufrêne and Legendre (1997) developed the Indicator Value Analysis (IndVal) as a statistically valid method for determining indicator species and species assemblages. IndVal considers the specificity, which is the mean number of individuals of a species across sampling sites of a certain habitat in comparison with other habitats, and the fidelity, described as the relative frequency of occurrence of a species in the sampling sites of a specific habitat. The IndVal analysis represents an efficient method to identify indicator organisms and can provide critical information on the ecological integrity of an ecosystem (Gardner 2010, McGeoch and Chown 1998). The IndVal approach has found application in various terrestrial and aquatic studies to assess habitat quality based on both macro- (Bazelet and Samways 2011, McGeoch et al 2002, Muotka et al 2002) and microorganisms (Auguet et al 2010, Fortunato et al 2013, Glasl et al 2016).

The integration of biological indicators into ecosystem monitoring programs can provide significant advantages. For example, by focusing on a targeted group of species, monitoring can become more cost and time efficient. Furthermore, biological indicators can provide early warning of environmental stress and might directly reveal the cause rather than simply the existence of a disturbance event. However, the choice of appropriate species or species assemblages is crucial for the effectiveness of bioindicators and hence, the selection should be based on sound quantitative approaches including consideration of the natural *in situ* variability (Carignan and Villard 2002).

Microorganisms as bioindicators to assess ecosystem health

The potential value of microorganisms for efficiently monitoring ecosystem health remains largely unexplored, despite the fact that microbes show fast, specific responses to environmental perturbations (Teeling et al 2012, Wemheuer et al 2015) which are the desirable characteristics of bioindicators (Table 1.1). One area where microorganisms have been exploited as indicators is in monitoring contamination of water supplies by coliform counts associated with faeces from warm-blooded animals (Ashbolt et al 2001). Coliform counts are now used to monitor and manage drinking water supplies and recreational water qualities worldwide (Boehm and Sassoubre 2014), successfully enabling the prevention of numerous human gastrointestinal illnesses (Wade et al 2010). In coral reef environments, coral mucus has been shown to be more efficient in trapping faecal indicator bacteria and human enteric viruses than the surrounding seawater; hence, coral mucus provides an improved medium to monitor sewage contamination (Lipp and Griffin 2004). Furthermore, seagrasses have recently been shown to reduce the abundance of microbial pathogens entering nearby coral reef and mitigate the disease risk in associated coral populations (Lamb et al 2017).

Intensive research on the structural and functional capacity of the human microbiome has revealed its enormous contribution to pathogenesis and immune system modulation of the host, as well as its influence on host development and physiology (Arrieta and Finlay 2012, Eloe-Fadrosh and Rasko 2013, Ghaisas et al 2016, Kostic et al 2015). This knowledge has revolutionized our current understanding of host-microbe interactions and has led to the development of diagnostic and therapeutic approaches targeting the human microbiome (Reardon 2014). For example, transplantation of faecal microbiomes has successfully cured humans affected by recurrent *Clostridium difficile* infections and microbiome transplantation has been suggested as a therapeutic cure for inflammatory bowel disease, obesity and HIV (Gupta et al 2016, Kang and Cai 2018, Mattila et al 2012, Nicholson et al 2018). In the same way, it is feasible that microbiome manipulation and probiotic treatment could be used to increase the health and tolerance of other reef based host-associated systems (Reshef et al 2006, van Oppen et al 2015, Webster and Reusch 2017). Also, similar to the approach in humans, the identification of imbalances in the microbial communities (dysbiosis) at the ecosystem level can facilitate diagnostic interpretations of environmental health. For example, a bacterial community based index has recently been developed to assess the ecological status of estuarine and costal environments (Aylagas and Rodriguez-Ezpeleta 2016). Another recent example applied to coral reef ecosystems has been the development of microbialisation scores, which attempt to assess human impacts on coral reefs based on the metabolic rates of microbial communities and reef-associated fishes (McDole et al 2012). While microbialisation has been shown to occur on a global scale (Haas et al 2016), additional

research is needed to adopt the approach to individual reef ecosystems. Microbial monitoring has also recently been introduced to the monthly sampling program of the National Mooring Network of IMOS (Integrated Marine Observing System), which targets oceanographic phenomena in Australian coastal waters (IMOS 2016). Increased appreciation of microorganisms in host and/or ecosystem health, together with recent advances in molecular techniques, now allow for detailed *in situ* investigations of the microbial community structure and functions and for standardized and efficient data analysis.

Changes in microbial communities due to disturbance may directly affect ecosystem processes. Therefore, it has been suggested to include microbial community composition (e.g. 16S ribosomal RNA gene sequencing) into process models that predict ecosystem responses to global change (Allison and Martiny 2008). Microbial functional redundancy is also of particular interest since metabolic capabilities can be decoupled from the phylogenetic position of microorganisms due to convergent evolution, gene loss or horizontal gene transfer (Martiny et al 2013, Ochman et al 2000). Phylogenetic diversity is thought to positively affect the stability of an ecosystem, as it increases the probability that complementary functional traits are present (Yachi and Loreau 1999). However, ecosystem processes can remain constant after disturbances even when a compositional shift has been observed (Allison and Martiny 2008, Banerjee et al 2016, Wohl et al 2004). Hence, functional approaches (e.g., metagenomics, metatranscriptomics) are also required for monitoring and predicting ecosystem changes. Functional and compositional changes can be assessed based on the entire community or on selected microbial indicator taxa.

Microbial life in the coral reef ecosystem

Within coral reefs, microorganisms colonize various habitats including the water column, the sediment and the benthic community, such as corals, sponges and macroalgae (Barott et al 2011, Bourne and Webster 2013, Rohwer et al 2001). The enormous complexity of coral reefs and their associated microbial communities has resulted in studies focused on these specific compartments; however, these habitats should not be considered as isolated from each other but rather seen as parts of a single ecosystem with a strong benthic-pelagic exchange (Garren and Azam 2012, Lesser 2006). Holistic approaches that combine the different reef habitats are urgently required to better understand the function and contribution of microorganisms to reef health and resilience.

Microbial diversity and function in coral reef waters

Microorganisms are moderately abundant (average densities of between 3 to 9 x 10^5 cells ml^{-1}) in nutrient-poor coral reef waters, where they play diverse roles related to nutrient cycling that ultimately affect the entire reef ecosystem (Ducklow 1990, Gast et al 1998, Sorokin 1973, Sorokin 1978). For instance, photoautotrophic picophytoplankton significantly contributes to the biomass and primary productivity of oligotrophic reef waters (Stockner 1988). Additionally, as part of the marine microbial-loop, heterotrophic bacteria utilize dissolved organic matter (DOM) in the water column, establishing an important recycling step that makes energy available to higher trophic levels (Azam et al 1983). In marine environments DOM is primarily of phytoplankton origin, as 2-50% of the photoautotrophically fixed carbon leaks into the water column (reviewed by Thornton 2014). Coral mucus, fuelled by the photosynthetic activity of the coral's algal symbionts, additionally contributes to the DOM pool of coral reef waters (Wild et al 2004a). The fixation of inorganic nitrogen is another key microbial function in coral reef environments, where nutrients are scarce and thus limit growth (Charpy et al 2012).

Processes shaping the microbial communities associated with coral reef waters vary over space and time. Distinct microbial communities along different niches within coral reef waters are the result of habitat structure, the presence of benthic host organisms and local biogeochemical conditions (Tout et al 2014). Seasonal effects, such as temperature, rainfall and water quality affect microbial community composition in shallow water reef sites (Angly et al 2016). Anthropogenic impact (e.g., land-use and fishing) also affects coral reef microorganisms with disturbed reefs possessing higher microbial abundances and a larger proportion of microbial taxa related to potential pathogens (Dinsdale et al 2008). The replacement of macro- with microorganisms under increased human influence has been referred to as microbialisation (Jackson et al 2001). Based upon the microbialisation concept, McDole et al (2012) developed the 'microbialisation score', a metric to assess the level of human impact and compare the health of coral reefs across time and space.

Microbial diversity and function in coral reef sediments

Coral reef sediments are typically dominated by calcareous sand, characterized by high permeability, porosity and surface area (Rasheed et al 2003). This enables large numbers of microorganisms to settle and grow on the sediment grains with prokaryotic abundance estimated at between 1 to 2 x 10^9 cells cm^{-2} of sediment surface (Wild et al 2006). In addition, the microbial communities associated with reef sediments are highly diverse, with vertical community stratification caused by redox gradients (Rusch et al 2009). The oxygenated upper sediment layer is dominated by heterotrophic processes and provides an important recycling step for dissolved and particulate organic matter (Wild et al 2004a). Wild et al (2004b) demonstrated that sloughed coral mucus acts as an efficient particle trap in the water column

and rapidly carries nutrients to reef sediments, where diverse microbial metabolisms successfully remineralise them. Fixation of inorganic nitrogen is also mediated by bacteria and archaea present within coral reef sediments and can substantially contribute to the overall coral reef nitrogen budget (Cardini et al 2014).

Importantly, Uthicke and McGuire (2007) identified clear bacterial community differences in surface sediments collected from inshore and offshore locations on the GBR and proposed they be used as biological indicators for water quality. Furthermore, a study conducted in the Red Sea has shown a nutritional link between seasonal dynamics and sediment-associated bacterial communities (Schöttner et al 2011). In addition to their metabolic functions, sediment associated microbes may also act as a seed-bank for microbes associated with coral mucus (Carlos et al 2013, Glasl et al 2016), another example of the tight microbial connections established within the coral reef ecosystem.

Corals and their microbiomes

Coral holobionts (Rohwer et al 2002) comprise an array of microorganisms including fungi, endolithic algae, bacteria, archaea and viruses (Koren and Rosenberg 2006, Ritchie and Smith 1997, Rohwer et al 2002, Sharshar et al 1997, Thurber et al 2008). The coral host itself provides several microhabitats for its microbial associates, such as the surface mucus layer (Frade et al 2016, Rohwer et al 2002), the tissue (Bourne and Munn 2005), the skeleton (Sharshar et al 1997) and the gastrovascular cavity (Herndl and Velimirov 1985). Each of the microhabitats within a coral colony is associated with a distinct microbial community (Bourne et al 2016, Rohwer et al 2002, Sweet et al 2011).

Bacterial communities associated with corals are clearly distinct from the bacterial communities in the surrounding environment (Frias-Lopez et al 2002) and bacterial cell abundance within coral mucus is also 10-fold higher than in seawater (Garren and Azam 2010). Each coral species serves as a unique habitat and is associated with a specific microbial community (Sunagawa et al 2010), with some species maintaining stable microbiomes over large geographic scales (Rohwer et al 2002), suggesting that the coral host plays a key role in structuring its bacterial community.

To deal with the enormous diversity of microorganisms associated with corals, and separate beneficial and opportunistic bacteria and archaea from the bulk microbial community, understanding coral holobiont stability and functionality is paramount. For instance, the mucus microbiome of healthy *Porites astreoides* colonies is dominated by *Endozoicomonadaceae*, whose loss is coupled to deterioration in holobiont health (Glasl et al 2016, Meyer et al 2014). *Endozoicomonas* also dominate the microbiome of *Stylophora pistillata* and *Pocillopora verrucosa* tissues; *Endozoicomonas* genotypes vary over geographic space in *S. pistillata,*

whereas in *P. verrucosa* the genotype of these endosymbionts remains the same. The genotype specificity may relate to differences in the life history strategies of corals (Neave et al 2017a). Based on meta-analysis of the core microbiomes of diverse coral species, members of *Actinobacteria* were recently identified as ubiquitous symbionts of corals (Ainsworth et al 2015). A decrease of *Actinobacteria* in coral microbiomes during periods of temperature and algal stress further suggests their beneficial role in the coral holobiont (Zaneveld et al 2016).

Potential functions of the coral's microbiome vary from nutrient supply and recycling (Lesser et al 2004, Lesser et al 2007) to protection against pathogens (Raina et al 2016, Ritchie 2006, Rohwer et al 2002, Shnit-Orland and Kushmaro 2009). For example, the depletion of the coral's beneficial mucus microbiome affected holobiont health under *in situ* conditions (Glasl et al 2016). This suggests that major disturbances of the surface mucus microbiome may open a niche for potentially opportunistic and/or pathogenic bacteria, which can further lead to diseases or host mortality. Recent investigation of the functional capabilities of the bacterial and archaeal community associated with corals revealed carbon fixation and degradation pathways and the presence of genes involved in sulphur and nitrogen cycling (Kimes et al 2010, Rädecker et al 2015, Siboni et al 2008 , Wegley et al 2007, Yang et al 2013). Nitrogen fixation capabilities are of particular interest as coral reefs are nitrogen-limited ecosystems (Falkowski et al 1993). Until recently, Cyanobacteria were thought to be the primary suppliers of fixed nitrogen in the coral holobiont (Lesser et al 2004, Lesser et al 2007). The discovery of the ubiquitous association with highly host specific diazotrophs (nitrogen fixing microorganisms) suggests their important functional role in the nitrogen supplementation of the coral holobiont (Lema et al 2012, Lema et al 2014, Siboni et al 2008), especially when external nitrogen sources are limited (Cardini et al 2015). Nitrifying, denitrifying and ammonia oxidizing microbes have also been described to be associated with corals; however, their precise functions within the holobiont remains understudied.

The microbiome of corals has been described as persistent over space and time (Rohwer et al 2002). However, compositional and functional shifts have been observed in association with seasonal environmental variation (Li et al 2014), host mucus-shedding dynamics (Glasl et al 2016), thermal stress (for example coral bleaching; Bourne et al (2008)), disease (Meyer et al 2014, Séré et al 2013), coral's proximity to macroalgae (Barott et al 2012, Sweet et al 2013) and increasing environmental pollution (Garren et al 2009, Kelly et al 2014, Ziegler et al 2016). For example, thermal stress leads to destabilization of coral-algae interactions (Brown 1997) and a shift towards an opportunistic and/or pathogenic microbial community (Ainsworth et al 2008, Bourne et al 2008, Littman et al 2011). Water quality changes associated with increased sediment and nutrient run-off can increase microbial abundance (D'Angelo and Wiedenmann 2014, Dinsdale et al 2008), coral disease frequency

(Bruno et al 2003, Thurber et al 2014) and higher macroalgal abundance on coral reefs (Kline et al 2006). Macroalgae exudates stimulate bacterial activity and copiotrophic bacterial growth, and cause shifts in the coral microbiome attributed to increased labile DOC and toxic secondary metabolites (Barott et al 2012, Haas et al 2016, Morrow et al 2011, Nelson et al 2013, Sweet et al 2013, Vega Thurber et al 2012). Environmentally induced changes in the coral microbiome generally result in higher microbial abundance and a shift away from beneficial microbes towards opportunistic and/or pathogenic bacterial taxa, such as *Vibrionaceae* and *Rhodobacteraceae* (Bourne et al 2016, Rothig et al 2016, Vega Thurber et al 2009, Ziegler et al 2016). Increased nutrient run-off in combination with reduced grazers (hence higher algal abundance) destabilizes the coral microbiome with detrimental consequences for the host, particularly when exposed to additional stressors such as parrotfish bites and thermal stress (Zaneveld et al 2016). The resulting imbalance in the holobiont composition (dysbiosis) can lead to functional changes of the microbiome and facilitate disease development or alterations in metabolism and/or immunity that lead to bleaching and/or necrosis, and ultimately coral death (reviewed by Thompson et al 2015).

Sponges and their microbiomes

Marine sponges (phylum: Porifera) are a highly diverse component of coral reefs, usually exceeding the diversity of corals and algae (Diaz and Rützler 2001). Sponges have the ability to filter up to 50,000 times their own volume every day (Reiswig 1971a) and due to this active suspension feeding they play a key role in benthic-pelagic coupling, thus providing a vital trophic link between the benthos and the ambient seawater (Gili and Coma 1998, Southwell et al 2008). Sponges are primarily considered to feed on picoplankton (0.2-2 µm) (Hanson et al 2009, Pile et al 1997, Reiswig 1971b); however, several recent studies have shown that certain sponges are also able to assimilate dissolved organic matter (DOM) from the water column (De Goeij et al 2008, Mueller et al 2014, Rix et al 2016). Cryptic Caribbean sponges, for instance, transform DOM into particulate organic matter (POM) and thereby significantly contribute to the recirculation of nutrients in coral reefs, a phenomenon known as the sponge-loop (De Goeij et al 2013), which is likely mediated by microbes living within the sponge.

Sponges generally live in close association with a wide variety of microorganisms including bacteria, archaea, unicellular algae and protists. These microorganisms are often present in high abundance, accounting for up to 40-60 % of the total sponge volume and reaching cell abundances that exceed those in the surrounding seawater by several orders of magnitude (Hentschel et al 2006). Sponges with high microbial densities are referred to as 'high microbial abundance sponges' (Hentschel et al 2003, Vacelet and Donadey 1977), in contrast to marine sponges that harbor only a relatively small number of microorganisms and are referred to as 'low-microbial abundance sponges' (Hentschel et al 2003). The implications

of these diversity differences for host resistance and resilience remain elusive. In addition to the importance of microorganisms as a sponge food source (Pile et al 1996, Reiswig 1971b), microbial associates have also been reported to participate in a diverse range of interactions, including parasitism, commensalism and mutualism (reviewed by Taylor et al 2007) with significant implications for the ecology, biology and physiology of sponges (Bourne and Webster 2013, Thacker and Freeman 2012).

Microbes are generally located in the sponge mesohyl region, a layer of connective tissue where microbial cells are either freely occurring or enclosed within specialized bacteriocyte cells (reviewed by Taylor et al 2007). The mesohyl is also the place where phagocytosis of food particles (e.g. picoplankton) takes place; hence, host-symbiont recognition mechanisms must be in action to prevent the phagocytosis of symbiont cells (Nguyen et al 2014, Wehrl et al 2007, Wilkinson et al 1984). Furthermore, Archaea and *Cyanobacteria* have been found in the pinacoderm, the outer surface of sponges formed by epithelial cells (Webster et al 2001, Wilkinson 1980). Sponge-symbionts generally show high host species-specificity and only a few bacterial species seem to be ubiquitously present in sponges. Bacterial species associated with different sponges are more closely related to each other than to bacteria from the ambient water column and, hence, these monophyletic groupings of sponge symbionts are often referred to as 'sponge-specific sequence clusters' (SCs; Hentschel et al 2002, Schmitt et al 2012). Whereas about half of the SCs have been shown to be vertically transmitted from adult sponges directly to their offspring, the other half is thought to be horizontally acquired by each generation from the surrounding seawater (Webster et al 2010). Interestingly, the candidate phyla *Poribacteria*, which is highly abundant in various marine sponges over a wide geographic range (Fieseler et al 2004), was recently described as part of the rare seawater biosphere (Webster et al 2010). Webster et al (2010) proposed that members of the rare seawater biosphere might act as seed organisms for widely occurring symbiont populations.

Sponge symbionts are capable of diverse metabolic functions. They play a crucial role in carbon, nitrogen, sulphur and phosphorous cycling and are also fundamental for the synthesis of essential vitamins within the sponge holobiont (reviewed by Webster and Thomas 2016). Examples of the mutualistic nature of sponge symbiont associations include the nitrifying *Thaumarchaeota* and bacteria, which gain energy through the oxidation of ammonia, a sponge waste product (Bayer et al 2008, Mohamed et al 2010, Webster et al 2001). Besides nutrient cycling capabilities within the host, symbionts are also shown to significantly contribute to host defence via the production of secondary metabolites (Hentschel et al 2001, Kennedy et al 2007).

Research on the resistance and resilience of the sponge microbiome to environmental

stressors including local (e.g., sedimentation and nutrients) and global (e.g., elevated seawater temperature and ocean acidification) pressures has shown that responses are highly species-specific. For instance, a number of studies have revealed no compositional changes in the sponge-associated microbiome upon short-term nitrogen, temperature or sediment pulses, suggesting a highly resistant association between the sponge holobiont members (Luter et al 2012a, Luter et al 2014, Simister et al 2012). Similar observations were obtained during transplantation experiments of the sponge *Aplysina cavernicola*; light stressed sponges showed no changes in their bacterial community composition nor their production of secondary metabolites (Thoms et al 2003). More recent research has shown that whilst the microbiome of different heterotrophic sponge species remains stable under light attenuation, the microbiome of phototrophic species can be significantly affected by light availability (Pineda et al 2016). The symbiotic community associated with *Rhopaloeides odorabile*, a common Great Barrier Reef sponge, undergoes major changes in community structure, accompanied by host tissue necrosis, upon exposure to temperatures greater than 32°C (Webster et al 2008). A more recent metagenomic and metaproteomic analysis revealed that *R. odorabile* symbionts lose their metabolic functional potential during the early stages of heat stress and hence destabilize the sponge holobiont before visual signs of stress occur in the host animal (Fan et al 2013). A comparison of healthy and diseased *Ircinia fasticulata* individuals revealed a significant shift in the microbiome prior to mass mortality attributed to high seawater temperatures. The observed shift was suggested to negatively affect host fitness and resistance to environmental stress (Blanquer et al 2016). Numerous studies of sponge disease have reported a higher bacterial diversity in diseased tissue (Angermeier et al 2012, Blanquer et al 2016, Olson et al 2014, Webster et al 2008), suggesting that a dysbiotic microbiome rather than infection by a specific pathogen also has a role in the disease process. It is also noteworthy that some instances of ‘sponge disease’, do not involve detectable shifts in the composition of the microbial community (Luter et al 2010), although no studies have yet assessed whether symbiotic function is impacted during disease which may contribute to the observed declines in host health.

Macroalgae and their microbiomes

Macroalgae have always been present in healthy coral reef ecosystems in relatively low abundance (Bruno et al 2014). However, overfishing and eutrophication can facilitate a shift from coral dominated to algal dominated reefs (Hughes et al 2007). The increase of macroalgal abundance on coral reefs is a threat for corals, not only because of direct competition for space but also because macroalgae have been shown to facilitate coral disease outbreaks, increase mortality and prevent larval settlement (Nugues et al 2004, Smith et al 2006, Sweet et al 2013, Webster et al 2015). Furthermore, macroalgae significantly alter their ambient environment by

releasing higher amounts of labile Dissolved Organic Carbon (DOC) compared to corals. This elevated DOC supports higher microbial growth rates, leads to a depletion of bioavailable DOC in seawater and facilitates the growth of copiotrophic and potentially pathogenic microbial taxa (Haas et al 2011, Haas et al 2016, Nelson et al 2013). These processes have been summarized in the DDAM model (DOC, disease, algae, microorganism; Barott et al 2012), which describes the feedback of macroalgae-derived labile DOC and how it fuels the less efficient metabolism of copiotrophic and pathogenic microorganisms in coral reefs thereby contributing to increased microbial respiration and the local acidification of seawater (Haas et al 2016, Sweet et al 2013).

Besides their influence on the seawater microbiome, macroalgae themselves are holobionts and are associated with a highly diverse microbiota (Barott et al 2011). Bacterial densities on the algal surface vary between 10^2 to 10^7 cells cm^{-2} depending on the thallus section, host species and season (Armstrong et al 2000). The epibacterial community composition significantly differs from the community in the ambient seawater and shows high host specificity and temporal adaptation (Burke et al 2011a, Goecke et al 2013, Lachnit et al 2009, Lachnit et al 2011). In addition to the biofilm community on the surface of seaweeds, macroalgae harbor a specialized and stable endophytic bacterial community (Hollants et al 2011). The specificity of epibacterial communities associated with different macroalgae is currently under reconsideration; microbial functioning, rather than phylogeny, seems to be consistent within the holobiont (Burke et al 2011a, Burke et al 2011b).

The algal microbiome significantly contributes to host morphogenesis, health and defence. For example, morphological abnormalities were observed in various *Ulva* species when grown under aposymbiotic conditions (Nakanishi et al 1996, Provasoli and Pintner 1980, Singh et al 2011). Epiphytic bacteria, such as *Vibrio sp*. and *Pseudoalteromonas sp.*, provide inhibitory properties against various biofouling organisms and hence contribute to host defence against unwanted colonization (reviewed by Egan et al 2013). Additionally, epiphytic bacteria supply the algal host with key nutrients. Heterotrophic bacteria provide CO_2 to the photoautotrophic host, and *Cyanobacteria*, dominant members of the epibacterial community, provide fixed-nitrogen (de Oliveira et al 2013, Penhale and Capone 1981, Phlips and Zeman 1990). Recent genomic and metagenomic studies of seaweed-associated bacterial communities revealed a diverse genetic repertoire including genes for phosphorous, nitrogen and iron utilization (Burke et al 2011a, Fernandes et al 2011, Thomas et al 2008).

In general, little is known about the factors controlling the microbial communities in the surface biofilm of macroalgae. Bacterial richness in the biofilm of the kelp *Laminaria hyperborea* is reported to increase as the kelp ages (Bengtsson et al 2012) and microbial community succession seems to be influenced by stochastic processes (Burke et al 2011a,

Trias et al 2012). Furthermore, disturbances such as temperature stress are shown to disrupt algal-holobiont homeostasis, which can lead to a switch in bacterial communities from surface-associated commensals to opportunistic pathogens (Case et al 2011).

Methods to develop novel microbial indicators for coral ecosystem health assessment

High diversity, high functional complexity and low cultivability has historically limited our ability to understand the marine microbial realm. However, advances in culture-independent techniques along with next-generation sequencing (NGS) have revolutionized the study of microbial ecology (Schuster 2008). Accompanied by meta-omic approaches, NGS provides a practical tool for the efficient analysis of microbial communities *in situ* which will facilitate the identification of microbial indicators.

The first step towards identification of microbial indicators to assess coral reef health is analysis of the temporal and spatial variability of microbial communities associated with certain habitats and the subsequent definition of microbial baselines. Compositional baselines can be established with high-throughput sequencing of the 16S ribosomal RNA (rRNA) taxonomic marker gene of bacteria and/or archaea associated with environmental samples. 16S rRNA gene sequencing is a ubiquitously applied technique in diverse fields of coral reef research (Bourne et al 2013, Webster et al 2010), providing an inexpensive tool to establish compositional baselines associated with coral reefs. In parallel, metagenomic sequencing can be used to establish functional and compositional microbial baselines as this comprehensive approach uses shotgun sequencing to generate an overview of all genes present in an environmental sample (including 16S rRNA genes). Metagenomics has been successfully used to assess functional responses of the coral microbiome to various stressors (Kelly et al 2014, Littman et al 2011, Vega Thurber et al 2009) and is also frequently applied to understand the fundamental basis of the sponge (Fan et al 2013, Thomas et al 2010), macroalgae (Burke et al 2011a, Martin et al 2014) and planktonic (Dinsdale et al 2008, Haas et al 2016, Tout et al 2014) microbiomes.

Once baselines are established, the compositional and functional response of the microbial communities upon disturbance can be investigated. Taxonomic variation can be observed using 16S rRNA gene sequencing and compositional and functional shifts can be detected using metagenomic sequencing. However, metagenomics is limited in its ability to describe functional variations upon disturbance because of the natural discrepancy between genes that are present and genes that are actually being expressed (Wang et al 2015).

Metatranscriptomic sequencing overcomes this limitation by assessing the function encoded by mRNA sequences. Metatranscriptomics involves analysing near-real-time gene expression patterns by sequencing mRNA molecules and has emerged as a state-of-the-art tool to study community metabolism of free-living microbes in the open ocean (Hewson et al 2010, Poretsky et al 2009) and to obtain insights in cell-cell signalling, development and immune response of symbiotic interactions (Chun et al 2008, Ruby 2008, Sanders et al 2013, Stewart et al 2011). It has also been used to elucidate nutrient cycling and vitamin production pathways of a sponge holobiont (Fiore et al 2015). Furthermore, responses of the coral host and its associated microbiome to coral diseases have been investigated based on mRNA sequences (Arotsker et al 2016, Daniels et al 2015). However, a clear limitation of this method is the short lifetime of mRNA molecules; it only takes a few minutes before mRNA molecules are degraded within the cell (Pedersen et al 2011). Metaproteomics can characterize the protein signatures from microbial communities *in situ* and also provides a link between gene content and gene expression (von Bergen et al 2013). Thus, metaproteomic studies are often complemented by metagenomic data. For example, the combination of metagenomic and metaproteomic data on the sponge microbiome provided novel insights in the activity, physiology and interactions between sponge symbionts (Liu et al 2012) and revealed the functional role of microbes in the stability of the sponge holobiont under thermal stress (Fan et al 2013).

Finally, a quantitative approach that establishes links between the composition/function of reef microbiomes and environmental metadata will be required to identify microbial indicators for coral reef health and water quality. Individual microbial taxa and/or functions significantly associated with healthy *versus* stressed reef systems can be identified using traditional statistical approaches such as the IndVal analysis (Dufrêne and Legendre 1997). An alternative approach to analysing meta-omics data and discriminating between healthy and stressed reef ecosystems is machine learning. Instead of the identification of individual microbial indicators, the entire microbial community and its function could be used to train a model to differentiate between reef health stages or environmental stressors. Machine learning is a powerful tool and a current state-of-the-art approach to identify dysbiosis of the human microbiome and to predict human diseases (Pasolli et al 2016).

Conclusion and future research

Microorganisms are fundamental contributors to reef ecosystem health through their biogeochemical capabilities and intimate symbiotic partnerships. Shifts in the composition or function of bacterial and archaeal communities can therefore provide crucial diagnostic information for future coral reef monitoring. Before such approaches can be developed and

implemented, the following basic questions need to be resolved (see Figure 1.2): i) which taxa and functions form the microbial baseline of healthy coral reefs? ii) how does the microbial community respond to environmental change? iii) how does the microbiome influence holobiont resistance and resilience upon disturbance? and iv) are environmental disturbances predicted by compositional and functional changes in the microbial community?

Regular monitoring is a fundamental tool for conservation and resource management of marine ecosystems in both developed and developing countries. For example, in the Australian GBR extensive reef monitoring records coral abundance, disease prevalence and coral cover, with the overarching objective of determining the status of reef health and pinpointing changes in the distribution and abundance of the reef biota over large temporal and spatial scales (AIMS 2017). Extending already existing monitoring initiatives to include sample collections targeting microbial biodiversity, composition and function would provide a cost-effective strategy to establish the first microbial reference datasets for individual reef locations (Phase 1). By combining microbial community data and other environmental parameters (e.g. water quality), microbial indicators (taxa or functions) can be identified allowing for reef health diagnosis (Phase 2). Once microbial indicators have been determined, the development and testing of cost- and time-efficient microbial monitoring protocols can begin (Phase 3). The ultimate goal of Phase 3 is the establishment of reliable, fast, low-cost and easy-to-use diagnostic protocols based on microbial indicators that can be integrated into current monitoring programs. Microbial monitoring protocols can be comprised of targeted PCR-based approaches (e.g., PCR screening for the occurrence of a specific microbial taxon/function, or quantification of its abundance) and/or community sequencing approaches (e.g. amplicon sequencing to track shifts in microbial community composition). The final step (Phase 4) is the integration of microbial indicators into standard reef monitoring procedures. A prospective microbial indicator tool kit will offer streamlined procedures covering sample collection and processing, an online data analysis platform, and recommended guidelines for management interventions based on the diagnosed reef conditions.

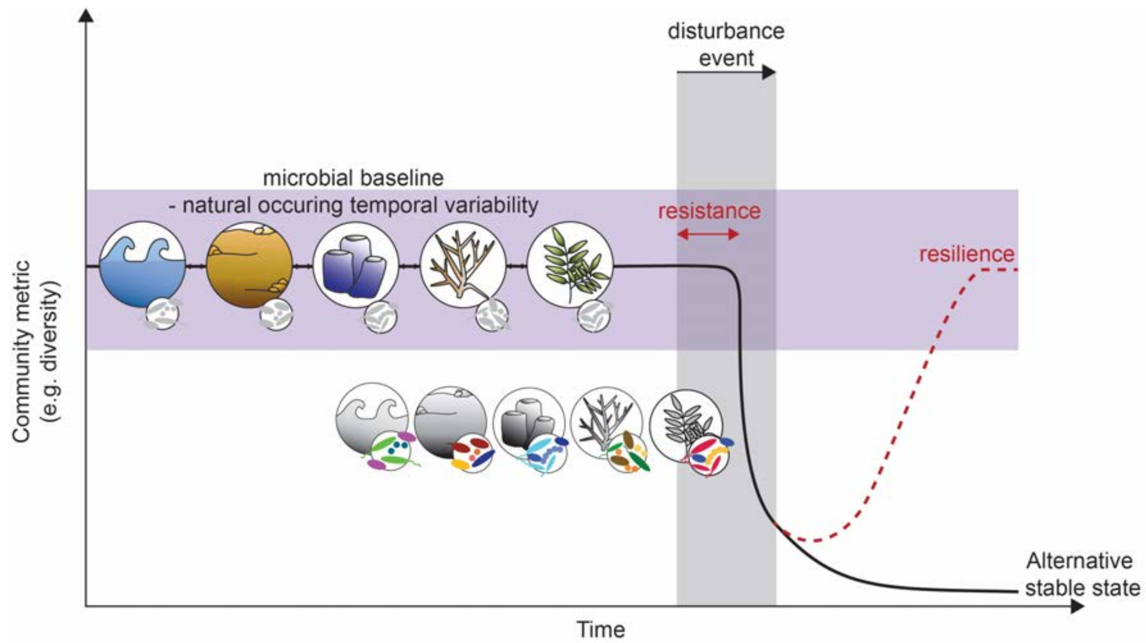

Figure 1.2. Schematic representation of the outlined future research directions: The microbial baseline (horizontal bar) needs to be defined for a comprehensive range of coral reef habitats (including seawater, sediment, sponges, corals and macroalgae), over temporal periods for a particular microbial community parameter of interest (e.g. diversity). A disturbance event (vertical bar) alters the environmental conditions, and triggers a shift in the microbial community associated with each habitat (solid black line) leading to an alternative stable state that may significantly deviate from the natural state. The stability of a holobiont, which relates to its ability to act as a buffer to maintain coral reef function upon environmental alteration, is here defined by the combined resistance and resilience of the microbial community. Resistance is the ability of a community to withstand a disturbance without change. Resilience is defined as the rate at which a microbial community regains its original state after a disturbance event. Combining the information on microbial community stability and variation will enable identification of microbial indicators leading to the early identification of imminent environmental stressors (e.g. water quality changes).

Chapter 2

ESTABLISHING MICROBIAL BASELINES TO IDENTIFY INDICATORS OF CORAL REEF HEALTH

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Abstract

Microorganisms make a significant contribution to reef ecosystem health and resilience via their critical role in mediating nutrient transformations, their interactions with macro-organisms and their provision of chemical cues that underpin the recruitment of diverse reef taxa. However, environmental changes often cause compositional and functional shifts in microbial communities that can have flow-on consequences for microbial-mediated processes. These microbial alterations may impact the health of specific host organisms and can have repercussions for the functioning of entire coral ecosystems. Assessing changes in reef microbial communities should therefore provide an early indicator of ecosystem impacts and would underpin the development of diagnostic tools that could help forecast shifts in coral reef health under different environmental states. Monitoring, management and active restoration efforts have recently intensified and diversified in response to global declines in coral reef health. Here we propose that regular monitoring of coral reef microorganisms could provide a rapid and sensitive platform for identifying declining ecosystem health that can complement existing management frameworks. By summarising the most common threats to coral reefs, with a particular focus on the Great Barrier Reef, and elaborating on the role of microbes in coral reef health and ecosystem stability, we highlight the diagnostic applicability of microbes in reef management programs. Fundamental to this objective is the establishment of microbial baselines for Australia's coral reefs.

Introduction

Coral reefs represent one of the most diverse ecosystems on the planet, providing home for an estimated 25 % of all known marine species (Connell 1978). Each year Australia's iconic Great Barrier Reef (GBR) attracts millions of tourists from all over the world and provides $ 6.4 billion dollars to the Australian economy (Deloitte 2017). However, reefs globally are facing unprecedented pressures (Hughes et al 2017a). During the past three decades, the GBR has also been severely impacted by the combined effects of climate change, crown of thorns starfish outbreaks, coral disease, overfishing and declining water quality (De'ath et al 2012, Hoegh-Guldberg 2011, Hughes et al 2017a). Back-to-back bleaching events were experienced in 2016 and 2017 on the GBR, resulting in over 80 % mortality of corals in some regions and an estimated loss of 29 % of corals across the GBR system (Great Barrier Reef Marine Park Authority 2017, Hughes et al 2017a). In addition to global pressures related to climate change, coral reefs are also affected at local scales (Knowlton and Jackson 2008). For example, the GBR is locally affected by the run-off from 35 river basins, draining an area of over 424,000

km^2 (Brodie et al 2012). Intensified agricultural land use in the GBR catchment area has caused an increase of sediments, nutrients and pesticides associated with terrestrial runoff, resulting in a significant decline in water quality which poses ongoing chronic and periodic acute threats to the health of the GBR (Waterhouse et al 2012).

Coral reef monitoring and management initiatives are well-established in Australia. For example, since the early 1980s the Australian Institute of Marine Science (AIMS) has assessed the health of Australia's coral reefs via its Long-Term Monitoring Program (LTMP). The Great Barrier Reef Marine Park Authority (GBRMPA) has managed the GBR area for over 40 years under the *Great Barrier Reef Marine Park Act 1975*. In 2015, the Australian and Queensland governments released the Reef 2050 Long-Term Sustainability Plan, outlining concrete measures to manage and protect the GBR over the next three decades. However, despite the focus on coral reef monitoring and management initiatives across all levels of government and strong community engagement in many areas, the coral reefs surrounding much of the Australian coastline, like other parts of the world, have demonstrated concerning declines in recent years (De'ath et al 2012, Hughes et al 2017a). One aspect that is poorly understood yet fundamental to coral reef functioning and ecosystem resilience is the contribution of microorganisms. Here we highlight that incorporating microbial based monitoring approaches into coral reef management initiatives will increase our understanding of reef ecosystem health and inform potential options for increasing reef resilience (Figure 2.1).

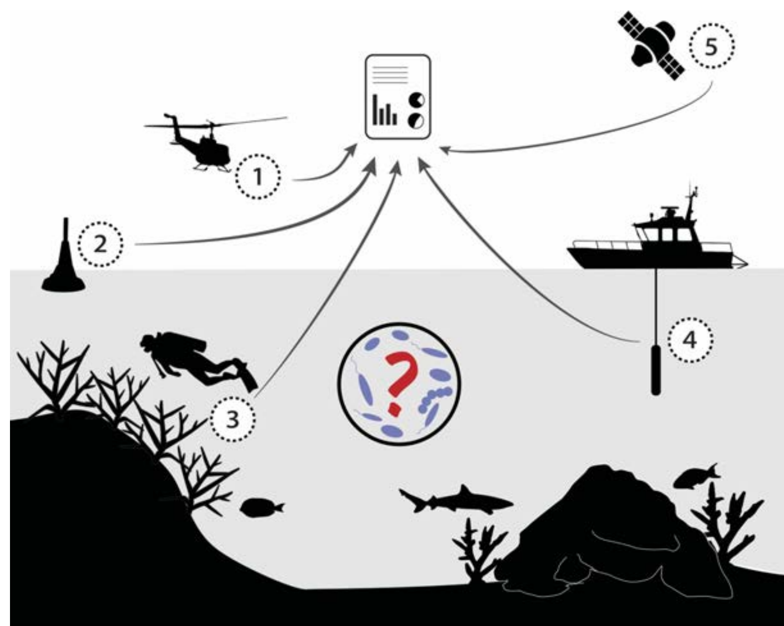

Figure 2.1. Implemented management strategies under Australia's Reef 2050 Long-Term Sustainability Plan are guided by an integrated monitoring approach including (1) large scale aerial surveys, (2) mooring systems and weather stations that provide data on surface (e.g., wind, precipitation, barometric pressure, temperature) and subsurface conditions (e.g., temperature, conductivity, chlorophyll fluorescence, turbidity, oxygen, light transmission and photosynthetically active radiation), (3) assessment of coral cover, coral recruitment, coral community composition and coral–macroalgae ratios on a reef, (4) comprehensive water quality assessments and screening for pesticide concentrations and (5) near surface concentration measurements of chlorophyll *a* and total suspended solids based on remote sensing technologies. Currently, this integrated monitoring framework lacks a microbial approach and hence, excludes a considerable part of the coral reef biodiversity.

Importance of microbes in coral reefs

Microorganisms play an essential role in coral reef ecosystem processes and form diverse symbiotic relationships with benthos-dominating macro-organisms such as corals, sponges and algae (Bourne et al 2016, Egan et al 2013, Webster and Thomas 2016; Figure 2.2). The functional role of microbes in coral reefs include biochemical cycling of nutrients, degradation and remineralisation, host nutrition, vitamin synthesis, production of secondary metabolites and host defence via the production of antimicrobial peptides (Bourne et al 2016, Webster and Thomas 2016). Microbes often form specific and stable associations with their host species (McFall-Ngai et al 2013) and can assist them to acclimate to the prevailing environmental conditions (Webster and Reusch 2017, Ziegler et al 2017).

Environmental variations, such as seasonal or anthropogenic-induced fluctuations in water quality are known to alter the composition and function of the reef microbiome (Angly et al 2016, Ziegler et al 2017). Numerous studies have shown a clear shift in microbial community composition and function in coral reef waters and associated with

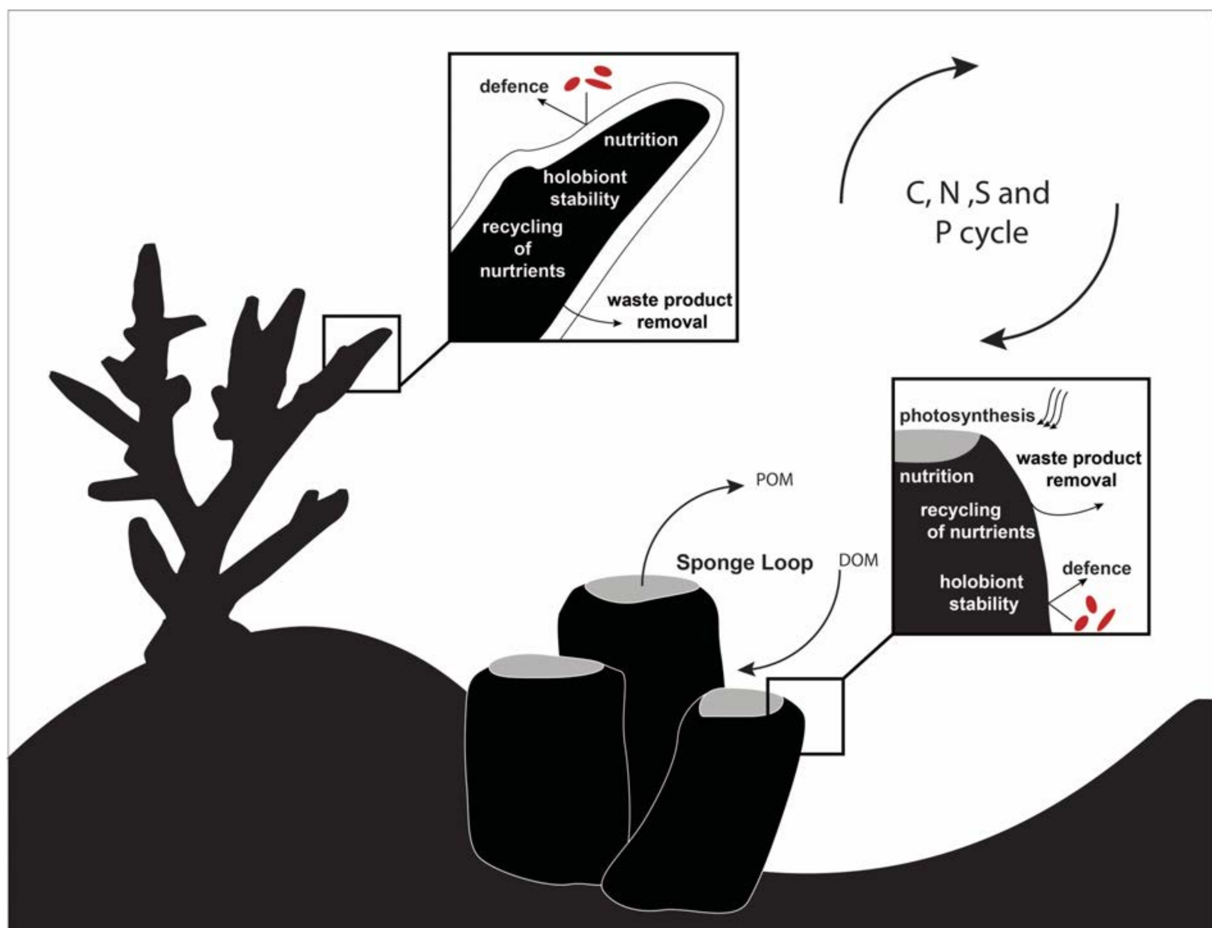

Figure 2.2. Simplified overview of microbial functions in a coral reef ecosystem. Microbes play a fundamental role in all major biogeochemical cycles (carbon, nitrogen, sulphur and phosphorus) in the coral reef ecosystem and contribute to their host's nutrition, waste product removal, pathogen defence and holobiont stability.

Figure 2.3. Coral reef ecosystems are increasingly affected by the intensification of environmental pressures emerging from land-use changes, overfishing, crown-of-thorns starfish outbreaks, coral diseases and climate change. Degradation of coral reefs and a shift from coral to macroalgae dominated benthic communities (from left to right) has been observed globally. As the health of the coral reef ecosystem changes, microorganisms rapidly respond. The microbiome of healthy reefs is dominated by beneficial and symbiotic microbes (blue), but as ecosystem health declines the microbiome shifts to an unbeneficial community, dominated by pathogens and opportunists (red).

dominant benthic life forms (such as corals) as the health of the ecosystem declines (Haas et al 2016, Zaneveld et al 2016; Figure 2.3). However, despite the recognized influence microbes have on coral reef health (Ainsworth and Gates 2016, Bourne et al 2016), a holistic understanding of their dynamics in coral reef ecosystems remains elusive (Garren and Azam 2012). Establishing microbial baselines that characterise the temporal and spatial microbial dynamics in coral reefs is urgently needed to underpin rapid and sensitive assessments of declining reef health and make predictions about the consequences of future environmental changes (Bourne et al 2016, Glasl et al 2017).

Australia's initiatives to establish microbial baselines

Recent advances in next generation sequencing technologies combined with an increased recognition of the crucial ecosystem roles played by microorganisms, has resulted in a heightened commitment to understand spatial and temporal microbial dynamics in Australian ecosystems. For example, the BASE project (Biomes of Australian Soil Environments) is the first Australian soil microbial diversity database, providing amplicon sequencing data alongside contextual data for more than 900 sites across Australia (Bissett et al 2016). Another example is the Australian Marine Microbial Biodiversity Initiative (AMMBI), which was the first standardized microbial ocean observatory program undertaken at a continental scale. AMMBI aims to provide long-term microbial sequencing data from seven different pelagic sites around Australia, providing important baseline data on microbial composition and function in

Australian off-shore waters. This is linked to extensive physicochemical and oceanography data derived from the Integrated Marine Observing System (IMOS) reference stations (www.imos.org.au), allowing both hindcasting and forecasting of microbial responses to environmental conditions. Recently the Marine Microbes (MM) project (www.bioplatforms.com/marine-microbes/) was established as part of the larger AMMBI initiative to sample microbial communities associated with corals, sponges, seaweeds, seagrasses, seawater and sediment from benthic sites across Australia, including sampling locations in the GBR, Perth and Sydney. The MM project aims to provide the first holistic microbial baseline for coral reefs in Australia.

Microbes as indicators of coral reef health

Indicator organisms are used to effectively monitor habitat conditions and environmental changes (De Cáceres et al 2010). Biological indicators are a well-established monitoring tool for estuarine and freshwater ecosystems (Smith et al 1999, Wright 1995) and also find application in coral reef ecosystems (Cooper et al 2009). In the context of public health, microorganisms are extensively used as indicators to monitor drinking water supplies and the quality of recreational waters in order to prevent gastrointestinal illnesses (Gruber et al 2014, Soller et al 2014). Furthermore, recent advances in human microbiome research have led to an increase of microbial based diagnostic and therapeutic approaches (Zmora et al 2016). Despite the emerging predictive power of the microbiome in human disease diagnostics (Knights et al 2011a, Zmora et al 2016), the use of microorganisms as sensitive indicators of environmental stress in coral reef ecosystems or as predictive markers for water quality in marine systems has remained relatively unexplored (Bourne et al 2016, Glasl et al 2017). Microbialisation scores are among the few attempts to monitor coral reef ecosystem declines based on the metabolic rates of microbial communities and reef-associated fishes (McDole et al 2012). Incorporating microbial monitoring tools into current coral reef health assessment programs will confer significant advantages as microbes are known to rapidly respond to changes in their environment, allowing for early diagnosis of changing water conditions and host physiological states.

Despite many potential advantages, microbial systems for monitoring coral reefs are still very much in their infancy and considerable additional research and validation would be required before microbial based monitoring approaches could be applied. Additional technical considerations that remain to be addressed include: 1) How frequently should sampling occur? 2) How and what should be sampled (e.g., seawater, sediment, microbiomes of benthic organisms such as corals or seaweed)? 3) What types of samples and analyses would be

necessary (e.g., community profiling, targeted screening for particular microbial indicator taxa and/or functions) and 4) How to minimise costs and increase efficiency of a microbial based monitoring system to ensure real-time assessment of reef health?

Conclusion

The important role of microbes in coral reef ecosystem functioning and their contribution to the resistance and resilience of coral reefs has become widely accepted (Ainsworth and Gates 2016, Glasl et al 2017). However, although Australia is at the forefront of coral reef studies and coral reef monitoring operations, to date, microbes have not been considered in large-scale monitoring approaches. The past few years have seen increased interest in understanding microbial dynamics in Australia's ecosystems which has led to holistic sampling efforts to establish the first microbial baselines for soils and marine environments. We argue that the establishment and ongoing assessment of such microbial baselines will be crucial to understanding microbial dynamics in response to broad ranging anthropogenic impacts. The inclusion of microbial monitoring approaches alongside our current coral reef monitoring framework will improve our ability to rapidly detect changes occurring in Australian coral reefs resulting in improved protection and management of these ecologically and economically unique ecosystems

Chapter 3

TAXONOMIC AND FUNCTIONAL BASELINES OF HOST-ASSOCIATED AND FREE-LIVING MICROBIAL COMMUNITIES FROM THE GREAT BARRIER REEF

Abstract

Microorganisms are fundamental members of coral reef ecosystems. However, despite their recognized ecological importance in coral reefs, large-scale microbial dynamics in the Great Barrier Reef (GBR) remain poorly understood. Lack of microbial baselines for the GBR represents a hurdle for understanding how well microbes respond to their surrounding environment and to pinpoint the diagnostic potential of microbial communities. To establish the first taxonomic and functional microbial baselines for multiple host-associated (i.e., coral, sponge, macroalgae) and free-living (i.e., sediment, seawater) microbial communities at selected GBR sites, monthly sampling over a 16-month collection period resulted in a total of 381 samples. The taxonomic composition of all microbial samples was assessed by amplicon based 16S rRNA gene sequencing using bacteria specific primer sets. A subset of the collected microbial samples was further used for metagenomic (n = 42) and metatranscriptomic (n = 36) sequencing. Microbial samples were collected and processed following standardised protocols established by the Australian Microbiome Initiative and all microbial data are freely available at the Bioplatforms Australia data portal.

Background and Summary

Over the past three decades the health of Great Barrier Reef (GBR) has been severely affected by the cumulative effects of climate change, crown-of-thorns starfish outbreaks, coral diseases, overfishing and declining water quality (De'ath et al 2012, Hughes et al 2017a). The back-to-back mass-bleaching events in 2016 and 2017, resulted in an unprecedented loss (approximately 29 %) of corals across the GBR (Hughes et al 2018a). The intensification of anthropogenic pressures in recent years and reports of wide-spread reef degradation emphasises the need for sensitive and effective coral reef monitoring and management initiatives.

Microorganisms are important members of coral reef ecosystems where they mediate biogeochemical cycling and form critical symbiotic partnerships with benthic organisms such as corals, sponges and macroalgae (Bourne et al 2016, Egan et al 2013, Webster and Thomas 2016). However, despite increasing awareness of the fundamental contribution of microorganisms to the functioning and resilience of coral reef ecosystems (Ainsworth and Gates 2016, Bourne et al 2016, Haas et al 2016), large-scale microbial datasets for the GBR remain scarce. Lack of microbial monitoring data for the GBR hinders our ability to assess changes in reef associated microbial communities in relation to overall coral reef ecosystem health (Glasl et al 2017, Glasl et al 2018a). Furthermore, the lack of microbial baseline datasets

has been highlighted as one of the major hurdles to successfully integrate microbial monitoring into the Reef 2050 integrated monitoring and reporting program (RIMReP) for the GBR.

In collaboration with the Australian Microbiome Initiative (https://www.bioplatforms.com/australian-microbiome/), the first methodologically standardised microbial baseline for the GBR was established. The Australian Microbiome Initiative is an Australian wide collaboration founded by two established Bioplatforms Australia framework data initiatives: the Marine Microbes and Biomes of Australian Soil Environments (Bissett et al 2016, Brown et al 2018). As part of the coastal component of the Marine Microbes framework data initiative, microbial baselines for corals, sponges, macroalgae, sediment and seawater from three inshore reefs in the central section of the GBR were established (Figure 3.1).

During a 16-month collection period (February 2016 until May 2017), 381 samples were collected at monthly (Geoffrey Bay, Magnetic Island) or periodic intervals (Pioneer Bay and Channel, Orpheus Island). The taxonomic composition of host-associated (i.e., coral, sponge, macroalgae) and free-living (i.e., seawater, sediment) bacterial communities was assessed using amplicon sequencing of the 16S rRNA gene using bacterial specific primer sets. The functional potential of seawater, macroalgae and sponge microbial communities was assessed using metagenomics, and gene-expression changes of the sponge microbiomes over a seasonal scale were further assessed using metatranscriptomics. These taxonomic and functional microbial data form the first comprehensive microbial baseline for the GBR spanning multiple host-associated and free-living microbial communities. Furthermore, this microbial baseline data provides a framework for future microbial observatories across the GBR and offers crucial insights into taxonomic and functional microbial dynamics in coral reef ecosystems over seasonal cycles.

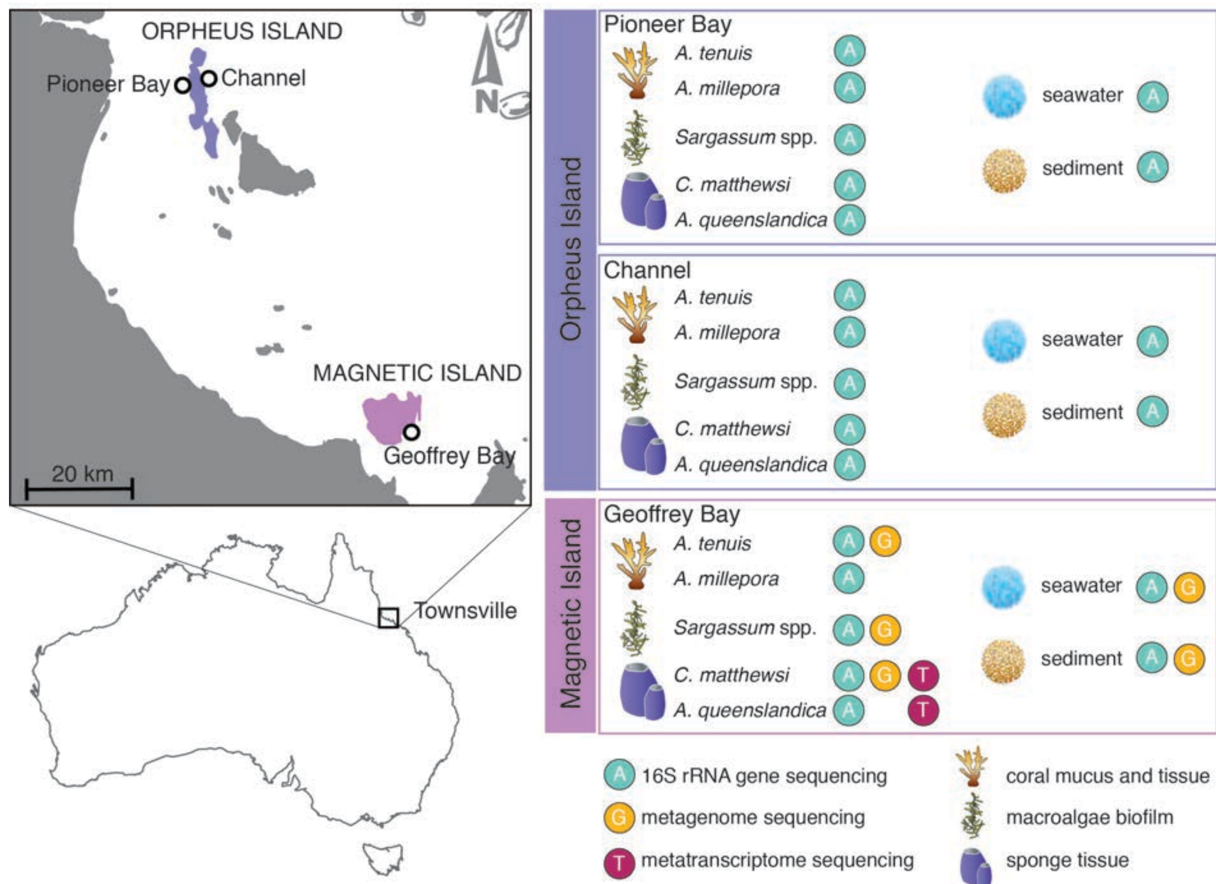

Figure 3.1. Overview of reef locations and microbial samples collected at the Great Barrier Reef as part of the Australian Microbiome Initiative. 16S rRNA gene sequencing data and metagenome sequencing data were analysed as part of this thesis. Samples for metatranscriptome sequencing were collected, processed and sequenced as part of this thesis.

Materials and Methods

Microbial sample collection

Microbial samples were collected monthly (Geoffrey Bay, Magnetic Island) and periodically (Pioneer Bay and Channel, Orpheus Island) from seawater and sediment, as well as benthic reef organisms such as corals, sponges and macroalgae between February 2016 and May 2017. Samples were collected under the permit G16/38348.1 by the Great Barrier Reef Marine Park Authority (GBRMPA). Microbial samples (n = 3 per sample type and sampling event) for meta-omic analysis were collected following the standard operational procedures of the Australian Microbiome Initiative (https://data.bioplatforms.com/organization/pages/australian-microbiome/methods) and the sampling procedures have recently been described by (Glasl et al 2019a). In detail, seawater samples were collected with collapsible sterile bags (5 L) at 2 m depth close to the reef substrate (approximately 50 cm) and pre-filtered through a plankton net (50 µm) to remove large particles. The pre-filtered seawater was subsequently filtered (2 L) onto 0.2 µm Sterivex-filters (Millipore) using a battery operated peristaltic pump (Pegasus

Alexis). Following filtration, both ends of Sterivex-filters were sealed with parafilm and packed individually into zip-lock bags. The sediment surface layer (~ 1cm) was sampled with sterile 50 mL tubes at 3 m depth. Immediately following collection, each sediment sample was subsampled into five 2 mL cryogenic vials (Corning) using sterile spatulas. Tissue (~ 30 cm^3) of the marine sponges *Coscinoderma matthewsi* (collected at 7 m depth) and *Amphimedon queenslandica* (3 m depth) were sampled using sterile scalpel blades, rinsed with 0.2 µm filtered-sterilised seawater and subsampled into five 2 mL cryogenic vials (Corning). The coral surface mucus of *Acropora millepora* (collected at 3 m depth) and *Acropora tenuis* (3 m depth) was sampled with sterile cotton swabs following published protocols (Glasl et al 2016). Three replicate mucus swabs were collected per coral colony. In addition to mucus, coral fragments (three fragments per colony) of each coral colony were collected, rinsed with 0.2 µm filtered-sterilised seawater and subsample into five 5 ml cryogenic vials (Corning). The thallus (~ 30 cm) of the macroalgae *Sargassum* spp. (including stem, floats and blades) was sampled with sterile scalpel blades at 3 m depth, rinsed with 0.2 µm filtered-sterilised seawater and subsampled into five 2 mL cryogenic vials (Corning). All microbial samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further processing.

Environmental metadata sample collection

Seawater for water quality analyses was collected at each sampling occasion with a diver-operated Niskin bottle at 2 m depth close to the reef substrate (approximately 50 cm). Seawater was subsampled into separate flasks: in duplicate for salinity (2 x 250 mL), dissolved organic carbon (DOC; 2 x 10 mL), particulate organic carbon (POC; 2 x 250 mL), dissolved inorganic nutrients (DIN; 2 x 10 mL), total suspended solids (TSS; 2 x 1 L), and chlorophyll *a* (Chl *a*; 2 x 250 mL). Sediment samples for grain size distribution (1 x 250 mL), total organic carbon (TOC) and total organic nitrogen (TON) content (1 x 250 mL) were also collected on each sampling occasion. Seawater and sediment samples were analysed by the Australian Institute of Marine Science (Townsville, Australia) following their implemented standard procedures (Devlin and Lourey 2000).

Sampling sites

Samples were collected at three inshore reef sites (Geoffrey Bay, Pioneer Bay and Channel). All sampled sites are fringing reefs surrounding continental islands (Magnetic Island and Orpheus Island) located in the central section of the GBR (Figure 3.1). An overview of reef locations, bioregions, sampling intervals, microbial samples collected and data produced by this project is provided in Table 3.1.

Table 3.1. Details of reef sites samples and microbial data collected for the establishment of microbial baselines for the Great Barrier Reef.

Reef site	Geographic position	GBR bioregion (Mellin et al 2019)	Microbial samples (hosts and habitats)	Sampling interval	Microbial data
Geoffrey Bay (Magnetic Island)	S 19°09.326', E 146°51.861'	Inshore macroalgae dominated reefs	*A. tenuis* (mucus and tissue) *A. millepora* (mucus and tissue) *C. matthewsi* *A. queenslandica* *Sargassum* spp. sediment seawater	Monthly (February 2016- March 2017)	Amplicon 16S rRNA (n= 253) metagenomes (n= 42) meta-transcriptomes (n = 36)
Pioneer Bay (Orpheus Island)	S 18°35.633’, E 146°29.052’	Inshore hard coral dominated reefs	*A. tenuis* (mucus and tissue) *A. millepora* (mucus and tissue) *C. matthewsi* *Sargassum* spp. sediment seawater	Periodic (May 2016- May 2017)	Amplicon 16S rRNA (n= 66)
Channel (Orpheus Island)	S18°34.612’, E146°29.816’	Inshore hard coral dominated reefs	*A. tenuis* (mucus and tissue) *A. millepora* (mucus and tissue) *C. matthewsi* sediment seawater	Periodic (May 2016- May 2017)	Amplicon 16S rRNA (n= 62)

Amplicon sequencing

DNA of seawater (n = 48), sediment (n = 48), sponge tissue (*C. matthewsi* n = 42, *A. queenslandica* n= 30), coral mucus (*A. tenuis* n= 46, *A. millepora* n = 42), coral tissue (*A. tenuis* n = 48, *A. millepora* n = 42) and macroalgae biofilm (*Sargassum* spp. n = 35) samples collected at Geoffrey Bay, Pioneer Bay and Channel was extracted for 16S rRNA gene sequencing. Samples of seawater, sediment, sponge and macroalgae were extracted using DNeasy PowerSoil kit (QIAGEN) and coral (tissue and mucus) samples were extracted using DNeasy PowerBiofilm kit (QIAGEN). Prior to DNA extractions, the macroalgal biofilm was separated from the *Sargassum* spp. thallus by overnight incubations at 200 rpm in 10 mL 1 x PBS at 37°C. Coral fragments were thawed on ice and the tissue was removed from the skeleton by airbrushing into 10 mL of 1 x PBS, homogenised for 1 min at 12,500 rpm with a hand-held tissue homogeniser (Heidolph Silent Crusher M), pelleted for 10 min at 16,000 rcf, and snap frozen in liquid nitrogen stored at -80°C until further processing.

DNA extracts were sent frozen (on dry ice) to the Ramaciotti Centre for Genomics (Sydney, Australia) for sequencing of the V1-V3 region of the 16S rRNA gene using the bacterial-specific primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’; Lane 1991) and 519R (5’-TTCCGGTTGATCCYGCCGGA-3’; Turner et al 1999). Libraries were prepared using the

TruSeq protocol, followed by sequencing on a Illumnia MiSeq platform using a duel indexed 2x 300 base pairs (bp) paired-end approach.

Sequencing data were analysed as zero-radius operational taxonomic units (zOTUS) via a standardised pipeline alongside other Australian Microbiome samples (Brown et al 2018). 1) Illumina forward (R1) and reverse (R2) reads were merged using FLASH (Magoc and Salzberg 2011). 2) FASTA formatted sequences were extracted from FASTQ files and sequences < 400 bp in length or containing N's or homopolymer runs of > 8 bp were removed using MOTHUR (v1.34.1) (Schloss et al 2009). 3) Sequences were de-replicated, ordered by abundance, and sequences with < 4 representatives were removed using USEARCH (Edgar 2010). 4) Chimeras were removed, biologically corrected, and zOTUs were identified. 5) Quality-filtered sequences (from step 2) were mapped to chimera-free zOTUs and a sample-by-read abundance table was created (note that chloroplasts and mitochondria derived reads were not removed). 6) 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.

Metagenome sequencing

DNA for metagenome sequencing was extracted from samples of seawater (n = 18), sediment (n = 6), sponge tissue (*C. matthewsi*, n = 6), coral mucus (*A. tenuis*, n= 6), and macroalgae biofilm (*Sargassum* spp., n = 6) collected at Geoffrey Bay. Seawater, sediment, and macroalgae samples were extracted using DNeasy PowerSoil kit (QIAGEN) and sponge samples were extracted using DNeasy PowerBiofilm kit (QIAGEN). Prior to extractions, the macroalgal biofilm was separated from the algal tissue by overnight incubations at 200 rpm in 10 mL 1 x PBS at 37°C. Microbes within sponge tissue were separated from sponge host cells as previously described by (Botte et al 2019). Loosely attached cells were removed from sponge tissue by washing the tissue twice (5 min at 200 rpm on an orbital incubation shaker) with sterile calcium- and magnesium-free seawater (CMFSW). The rinsed sponge tissue was homogenised using a handheld tissue homogeniser (Heidolph Silent Crusher M) for 10 min at 7,000 rpm in CMFSW. Next, 0.2μm filter sterilised collagenase (Sigma Aldrich) was added to the homogenised sponge tissue at a final concentration of 0.5 mg/mL and the tissue slurry incubated on ice for 30 min at 150 rpm on an incubation orbital shaker. After incubation, the sponge tissue slurry was filtered through a 100 μm sieve (In Vitro Technologies) into a sterile 50 ml tube. The filtrate was centrifuged at 100 rcf for 15 min at 4℃ and the resulting supernatant was subsequently centrifuged at 300 rcf for 15 min at 4℃. The recovered supernatant was filtered two-times through 8 μm filters, followed by two filtrations through 5 μm filters (Millipore). The final filtrate was centrifuged for 20 min at 8,800 rcf at 4℃. The resulting microbial pellet was washed two-times in 10 mL Tris/NaCl, centrifuged for 20 min at

5,000 g at 4°C and the final pellet was recovered in sterile 1mL Tris-HCl/NaCl.

DNA extracts for metagenome sequencing were shipped on dry ice to the Australian Genome Research Facility (AGRF; Melbourne, Australia). Libraries were prepared with the Nextera XT Library Preparation kit (Illumina), following the manufacturer's protocol and sequenced on an Illumina HiSeq 2500 in rapid run mode with 250 bp paired-end reads (24 samples per flow cell resulting in approximately 5 to 6 Gbp per sample).

Metatranscriptome sequencing

RNA for metatranscriptomic sequencing was extracted from sponge tissue samples (*C. matthewsi*, n = 18, and *A. queenslandica*, n = 18) collected at Geoffrey Bay following the RNA extractions procedures of the Australian Microbiome Initiative. Frozen sponge tissue was crushed on liquid nitrogen with a French pressure cell press. The total RNA of frozen sponge material was extracted following the Trixol extraction and DNase treatment protocol of the PureLink RNA mini kit (Thermo Fisher Scientific). The eukaryotic (i.e. sponge) and prokaryotic RNA was separated using Poly-A Purist Mag mRNA kit (Thermo Fisher Scientific). The prokaryotic RNA fraction was subsequently purified following the purifying RNA from liquid sample and purification protocols of the RNA mini kit (Thermo Fisher Scientific). Prokaryotic RNA was shipped on dry ice to the Australian Genome Research Facility (AGRF; Melbourne, Australia), where the rRNA was depleted using the Ribo-Zero rRNA removal kit (plant and bacteria probes) leading to mRNA enrichment. Libraries of enriched mRNA samples were subsequently prepared using Illumina TruSeq Stranded mRNA kits without poly(A) purification as per the manufacturer's instructions. Libraries were sequenced on an Illumina HiSeq 2500 platform in high output mode with 100 bp paired-end reads (10 samples per flow cell; ~ 20 million read pairs per sample).

Data Record and Usage Notes

Sequencing data (raw and processed), corresponding metadata and protocols are freely available upon registration at the Bioplatforms Australia data portal under the Australian Microbiome project (www.data.bioplatforms.com). Each microbial sample has a unique identifier (BPA ID) and a full list of microbial samples from the GBR is provided in Table 2 below. Sequence files (FASTQ format) can be downloaded individually (per sample) using the unique BPA ID. Amplicon sequencing data can be also downloaded by navigating to "Processed data", selecting "Amplicon is 27f519r_bacteria" and "Environment is Marine". To search for amplicon sequencing data originated from the GBR sites, add an additional contextual filter and select "Sampling Site" from the dropdown menu. This will enable searching

for the GBR specific sampling sites (Geoffrey Bay, Pioneer Bay, and Channel) and downloading of the associated sequence data.

Microbial samples, including their DNA and RNA (where applicable) extracts, are archived in multiple aliquots at -80°C at the Australian Institute of Marine Science (AIMS) in Townsville (Australia).

Table 3.2. Overview of individual microbial samples collected and microbial data available. BPA ID is a unique identifier for each microbial sample and can be used to extract sequencing data from the Bioplatforms Australia data portal (Data: A = amplicon sequencing data of the 16S rRNA gene, G = metagenome data, T = metatranscriptome data).

Microbial sample	Sample Site	Date sampled	BPA ID			Data		
seawater	Geoffrey Bay	2016-02-26	34848	34849	34850	A		
		2016-03-30	34851	34852	34853	A		
		2016-04-22	34854	34855	34856	A		
		2016-06-08	34863	34864	34865	A	G	
		2016-08-01	36308	36309	36310	A	G	
		2016-10-10	36311	36312	36313	A	G	
		2016-11-22	36320	36321	36322	A		
		2016-12-21	36323	36324	36325	A	G	
		2017-02-20	36326	36327	36328	A	G	
		2017-03-18	36329	36330	36331	A	G	
	Pioneer Bay	2016-05-02	34857	34858	34859	A		
		2016-11-09	36314	36315	36316	A		
		2017-05-06	36335	36336	36337	A		
	Channel	2016-05-04	34860	34861	34862	A		
		2016-11-10	36317	36318	36319	A		
		2017-05-08	36332	36333	36334	A		
sediment	Geoffrey Bay	2016-02-26	34830	34831	34832	A		
		2016-03-30	34833	34834	34835	A		
		2016-04-22	34836	34837	34838	A		
		2016-06-08	34845	34846	34847	A		
		2016-08-01	36341	36342	36343	A	G	
		2016-10-10	36350	36351	36352	A		
		2016-11-22	36353	36354	36355	A		
		2016-12-21	36356	36357	36358	A		
		2017-02-20	36359	36360	36361	A	G	
		2017-03-18	36338	36339	36340	A		
	Pioneer Bay	2016-05-02	34839	34840	34841	A		
		2016-11-09	36344	36345	36346	A		
		2017-05-06	36365	36366	36367	A		
	Channel	2016-05-04	34842	34843	34844	A		
		2016-11-10	36347	36348	36349	A		
		2017-05-08	36362	36363	36364	A		
A. queenslandica	Geoffrey Bay	2016-02-26	34881	34882	34883	A		
		2016-03-30	34884	34885	34886	A		
		2016-04-22	34887	34888	34889	A		
		2016-06-08	34890	34891	34892	A		T

	Geoffrey Bay	2016-08-01	36368	36369	36370	A		T
		2016-10-10	36371	36372	36373	A		T
		2016-11-22	36374	36375	36376	A		
		2016-12-21	36377	36378	36379	A		T
		2017-02-20	36380	36381	36382	A		T
		2017-03-18	36383	36384	36385	A		T
C. matthewsi		2016-03-30	34866	34867	34868	A		
		2016-04-22	34869	34870	34871	A		
		2016-06-08	34878	34879	34880	A		T
		2016-08-01	36386	36387	36388	A	G	T
		2016-10-10	36389	36390	36391	A		T
		2016-11-22	36398	36399	36400	A		
		2016-12-21	36401	36402	36403	A		T
		2017-02-20	36404	36405	36406	A	G	T
		2017-03-18	36407	36408	36409	A		T
	Pioneer Bay	2016-05-02	34872	34873	34874	A		
		2016-11-09	36392	36393	36394	A		
		2017-05-06	36410	36411	36412	A		
	Channel	2016-05-04	34875	34876	34877	A		
		2016-11-10	36395	36396	36397	A		
Sargassum spp.	Geoffrey Bay	2016-02-26	36413	36414	36415	A		
		2016-03-30	36416	36417	36418	A		
		2016-04-22	36419	36420	36421	A		
		2016-06-08	36422	36423	36424	A		
		2016-08-01	36425	36426	36427	A	G	
		2016-10-10	36428	36429	36430	A		
		2016-11-22	36433	36434	36435	A		
		2016-12-21	36436	36437	36438	A		
		2017-02-20	36439	36440	36441	A	G	
		2017-03-18	36442	36443	36444	A		
	Pioneer Bay	2017-05-06	36445	36446	36447	A		
	Channel	2016-11-10	36431	36432		A		
A. tenuis (tissue)	Geoffrey Bay	2016-02-26	36448	36449	36450	A		
		2016-03-30	36451	36452	36453	A		
		2016-04-22	36454	36455	36456	A		
		2016-06-08	36463	36464	36465	A		
		2016-08-01	36466	36467	36468	A		
		2016-10-10	36469	36470	36471	A		
		2016-11-22	36478	36479	36480	A		
		2016-12-21	36481	36482	36483	A		
		2017-02-20	36484	36485	36486	A		
		2017-03-18	36487	36488	36489	A		
	Pioneer Bay	2016-05-02	36457	36458	36459	A		
		2016-11-09	36472	36473	36474	A		
		2017-05-06	36490	36491	36492	A		
	Channel	2016-05-04	36460	36461	36462	A		
		2016-11-10	36475	36476	36477	A		
		2017-05-08	36493	36494	36495	A		
A. tenuis (mucus)	Geoffrey Bay	2016-02-26	36538	36539		A		

		2016-03-30	36540	36541		A	
		2016-04-22	36542	36543	36544	A	
		2016-06-08	36551	36552	36553	A	
		2016-08-01	36554	36555	36556	A	G
		2016-10-10	36557	36558	36559	A	
		2016-11-22	36566	36567	36568	A	
		2016-12-21	36569	36570	36571	A	
		2017-02-20	36572	36573	36574	A	G
		2017-03-18	36575	36576	36577	A	
	Pioneer Bay	2016-05-02	36545	36546	36547	A	
		2016-11-09	36560	36561	36562	A	
		2017-05-06	36578	36579	36580	A	
	Channel	2016-05-04	36548	36549	36550	A	
		2016-11-10	36563	36564	36565	A	
		2017-05-08	36581	36582	36583	A	
A. millepora (tissue)	Geoffrey Bay	2016-04-22	36496	36497	36498	A	
		2016-06-08	36505	36506	36507	A	
		2016-08-01	36508	36509	36510	A	
		2016-10-10	36511	36512	36513	A	
		2016-11-22	36520	36521	36522	A	
		2016-12-21	36523	36524	36525	A	
		2017-02-20	36526	36527	36528	A	
		2017-03-18	36529	36530	36531	A	
	Pioneer Bay	2016-05-02	36499	36500	36501	A	
		2016-11-09	36514	36515	36516	A	
		2017-05-06	36532	36533	36534	A	
	Channel	2016-05-04	36502	36503	36504	A	
		2016-11-10	36517	36518	36519	A	
		2017-05-08	36535	36536	36537	A	
A. millepora (mucus)	Geoffrey Bay	2016-04-22	36584	36585	36586	A	
		2016-06-08	36593	36594	36595	A	
		2016-08-01	36596	36597	36598	A	
		2016-10-10	36599	36600	36601	A	
		2016-11-22	36608	36609	36610	A	
		2016-12-21	36611	36612	36613	A	
		2017-02-20	36614	36615	36616	A	
		2017-03-18	36617	36618	36619	A	
	Pioneer Bay	2016-05-02	36587	36588	36589	A	
		2016-11-09	36602	36603	36604	A	
		2017-05-06	36620	36621	36622	A	
	Channel	2016-05-04	36590	36591	36592	A	
		2016-11-10	36605	36606	36607	A	
		2017-05-08	36623	36624	36625	A	

Chapter 4

MICROBIAL INDICATORS OF ENVIRONMENTAL PERTURBATIONS IN CORAL REEF ECOSYSTEMS

This chapter is published as

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Abstract

Coral reefs are facing unprecedented pressure on local and global scales. Sensitive and rapid markers for ecosystem stress are urgently needed to underpin effective management and restoration strategies. Although the fundamental contribution of microbes to the stability and functioning of coral reefs is widely recognised, it remains unclear how different reef microbiomes respond to environmental perturbations and whether microbiomes are sensitive enough to predict environmental anomalies that can lead to ecosystem stress. However, the lack of coral reef microbial baselines hinders our ability to study the link between shifts in microbiomes and ecosystem stress. In this study, we established a comprehensive microbial reference database for selected Great Barrier Reef sites to assess the diagnostic value of multiple free-living and host-associated reef microbiomes to infer the environmental state of coral reef ecosystems. A comprehensive microbial reference database, originating from multiple coral reef microbiomes (i.e., seawater, sediment, corals, sponges and macroalgae), was generated by 16S rRNA gene sequencing for 381 samples collected over the course of 16 months. By coupling this database to environmental parameters, we showed that the seawater microbiome has the greatest diagnostic value to infer shifts in the surrounding reef environment. In fact, 56% of the observed compositional variation in the microbiome was explained by environmental parameters, and temporal successions in the seawater microbiome were characterized by uniform community assembly patterns. Host-associated microbiomes, in contrast, were five-times less responsive to the environment and their community assembly patterns were generally less uniform. By applying a suite of indicator value and machine learning approaches we further showed that seawater microbial community data provide an accurate prediction of temperature and eutrophication state (i.e., chlorophyll concentration and turbidity). Our results reveal that free-living microbial communities have a high potential to infer environmental parameters due to their environmental sensitivity and predictability. This highlights the diagnostic value of microorganisms and illustrates how long-term coral reef monitoring initiatives could be enhanced by incorporating assessments of microbial communities in seawater. We therefore recommend timely integration of microbial sampling into current coral reef monitoring initiatives.

Introduction

Coral reef ecosystems are rapidly degrading due to local and global pressures (Hughes et al 2017a). Overfishing, pollution, declining water quality, disease and outbreaks of coral predating crown-of-thorns starfish are responsible for localised reef degradation (De'ath et al

2012) while climate change is impacting reefs on a global scale, including remote reefs with little local anthropogenic pressure (Hoegh-Guldberg et al 2007). For example, elevated sea surface temperatures caused back-to-back coral mass bleaching events in 2016 and 2017, resulting in a significant loss of shallow-water corals on the Great Barrier Reef (GBR) (Hughes et al 2018a). Climate conditions predicted for the end of the century will result in even more frequent and severe coral mass bleaching events with dire projections for the future of coral reefs (Hughes et al 2018b, van Hooidonk et al 2016). This global coral reef crisis is driving the development of new management, reef restoration and bioengineering tools to counteract reef loss and ensure the persistence of coral reefs (Anthony et al 2017, Damjanovic et al 2017). Early prediction of ecosystem stress is critical for an effective implementation of local management and restoration strategies on threatened reef sites.

Microorganisms have considerable potential as a monitoring tool for coral reef ecosystem health (Glasl et al 2017, Glasl et al 2018a, Roitman et al 2018). Microorganisms are fundamental drivers of biogeochemical cycling on coral reefs (Bourne and Webster 2013, Gast et al 1998, Sorokin 1973), they form intimate associations with the coral reef benthos (Egan et al 2013, Rohwer et al 2002, Webster et al 2012), and they contribute significantly to host health and ecosystem homeostasis (Glasl et al 2016, Hentschel et al 2001, Webster and Reusch 2017). The constant amendment of microbial communities to exploit available resources (Martiny et al 2015) can trigger differential abundances of specific microorganisms, hence shifts in community composition can provide an early indication of environmental change (Garza et al 2018). For example, compositional and functional shifts of coral-associated microbial communities have been described along gradients of anthropogenic impact (Dinsdale et al 2008, Kelly et al 2014, Ziegler et al 2016) and with changes in water quality (Angly et al 2016). However, despite having many of the useful characteristics required of environmental indicators (Cooper et al 2009, Glasl et al 2017), the diagnostic potential of microorganisms for coral reef monitoring is largely conceptual, with only a few studies elaborating on their potential value. For example, the ‘microbialisation score’ measures human impacts on coral reefs based on the ratio of microbial and fish metabolic rates (McDole et al 2012). The main limitations to further develop and apply microbial-based monitoring approaches are the lack of temporal and spatial baselines for coral reef microbiomes (Bourne et al 2016, Glasl et al 2017).

Coral reefs comprise a complex network of free-living and host-associated microbial communities with strong benthic-pelagic exchange (Bourne and Webster 2013, Lesser 2006). Therefore, holistic assessments that combine different reef hosts and habitats are required to better understand microbial dynamics and sensitivities to environmental perturbations. The diagnostic value of microbial-based monitoring is likely to vary between distinct habitats of a

coral reef ecosystem. For example, microbial communities occurring in seawater may be directly affected by the quality of the ambient reef water or climate conditions, however, the high heterogeneity of seawater due to local hot-spots of available resources (Azam 1998, Stocker 2012) may diminish the specificity of these communities. In contrast, microbial communities that dwell in corals live in tight association with the most important frame-builders of reefs (Bourne et al 2016) and hence may provide crucial information not only on the environmental conditions but also on the effect of the environment on the coral host itself. Sponges, a highly abundant and diverse component of coral reefs (Diaz and Rützler 2001), are renowned for their enormous filtration capacity (Reiswig 1971a) and form diverse and intimate associations with microbial communities (Taylor et al 2007). Hence, sponge microbiomes may provide suitable indicators to monitor water quality. Host-associated biofilms, such as those inhabiting the mucus layer of corals and the surface of macroalgae, provide another potential niche habitat informative for microbial indicators of environmental state. Coral mucus, for example, has been described as a suitable habitat to screen for enterobacteria from sewage contamination due to its ability to trap bacteria (Lipp and Griffin 2004).

Given the complexity of microbial life on coral reefs we sought to identify the most suitable reef microbiomes for a microbial indicator program to pinpoint environmental state. To do this we quantified the 1) habitat-specificity, 2) determinacy of microbial community successions and 3) sensitivity towards environmental parameters of multiple free-living and host-associated microbiomes. Subsequently, we tested the microbiome's ability to infer environmental state using indicator value (De Cáceres and Legendre 2009) and machine learning approaches (Knights et al 2011b).

Materials and Methods

Sample collection

Samples for microbial community characterization were collected monthly (Magnetic Island) and periodically (Orpheus Island) from seawater, sediment and multiple host organisms (i.e., corals, sponges and macroalgae), along with environmental metadata, between February 2016 and May 2017 at three Great Barrier Reef sites (Figure 4.1). Samples were collected under the permit G16/38348.1 issued by the Great Barrier Reef Marine Park Authority.

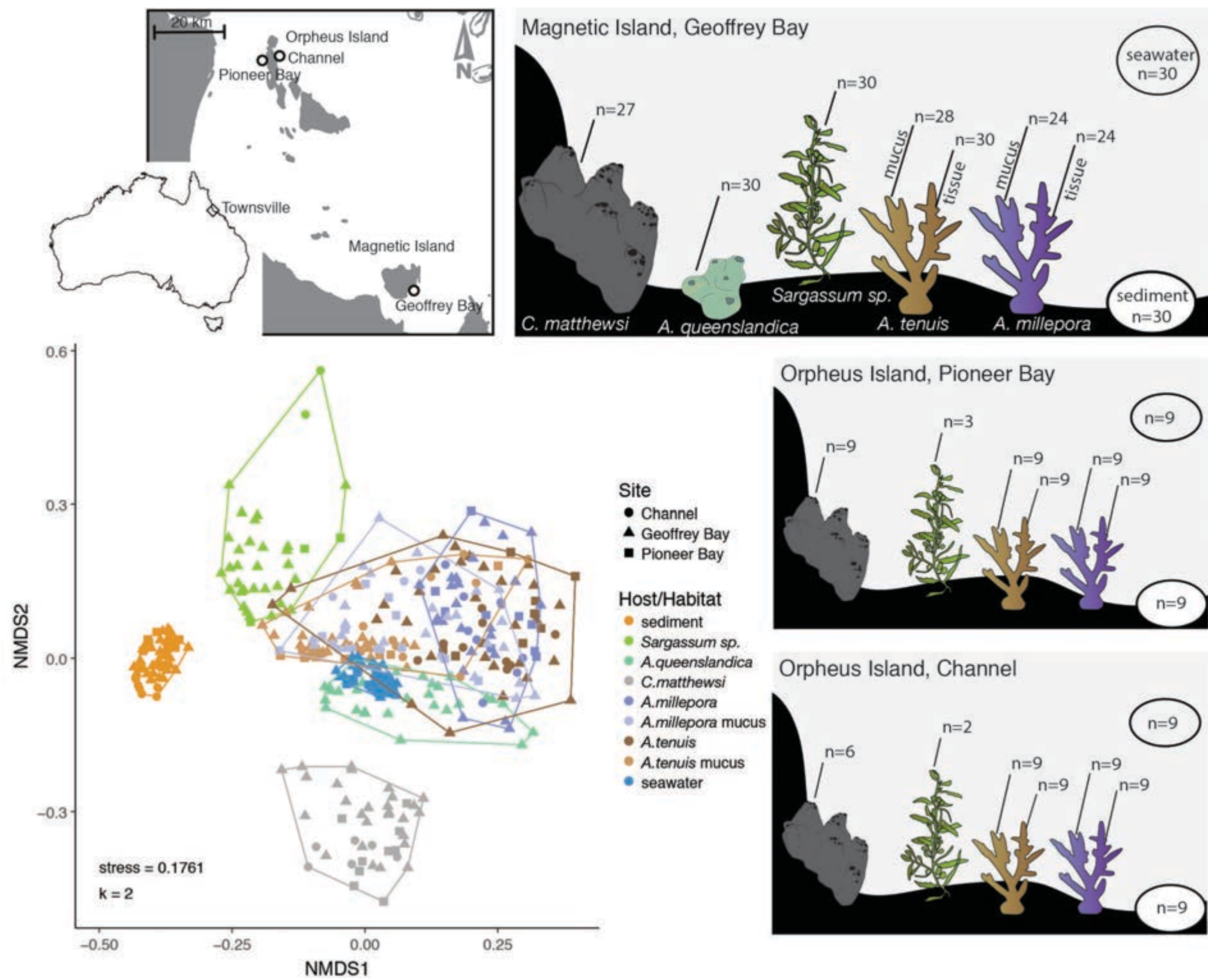

Figure 4.1. Habitat-specificity of coral reef microbiomes. Seawater, sediment, coral (*Acropora tenuis* and *Acropora millepora*), sponge (*Amphimedon queenslandica* and *Coscinoderma matthewsi*) and macroalgae (*Sargassum sp.)* samples were collected for 16S rRNA gene sequencing at fringing reefs surrounding Magnetic Island (Geoffrey Bay) and Orpheus Island (Pioneer Bay and Channel; Queensland, Australia). Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities revealed high habitat-specificity of coral reef microbiomes.

Samples (n= 3/ sample type/ sampling event) for molecular analysis and additional environmental metadata were collected following the standard operational procedures of the Australian Marine Microbial Biodiversity Initiative (AMMBI; https://data.bioplatforms.com/organization/pages/australian-microbiome/methods). In brief, seawater for molecular analysis was collected with collapsible sterile bags close to the reef substrate at 2 m depth and pre-filtered (50 µm) to remove large particles and subsequently filtered (2 L) onto 0.2 µm Sterivex-filters (Millipore). The sediment surface layer was sampled with sterile 50 mL tubes at 2 m depth and subsampled immediately into 2 mL cryogenic vials. The sponges *Coscinoderma matthewsi* and *Amphimedon queenslandica* were removed from the substrate (at 7 m and 3 m respectively) with sterile scalpel blades, rinsed with 0.2 µm filter-sterilised seawater and subsampled into 2 mL cryogenic vials. The surface mucus layer of the two acroporid coral species, *Acropora tenuis* and *Acropora millepora*, was sampled with sterile cotton swabs (Glasl et al 2016). Additionally, coral fragments of each sampled coral were

collected at 3 m depth. Coral fragments were rinsed with 0.2 µm filtered-sterilised seawater and placed into 5 mL cryogenic vials. The thallus (including stem, floats and blades) of the macroalgae *Sargassum sp.* was sampled with sterile scalpels at 3 m depth, rinsed with 0.2 µm filtered-sterilised seawater and placed into 2 mL cryogenic vials. All samples were immediately flash frozen in liquid nitrogen after processing and stored at -80°C until DNA extraction.

Additional seawater samples were collected with a diver-operated Niskin bottle close to the reef substrate at 2 m depth at each sampling occasion. Water was subsampled in duplicate for analyses of salinity and concentrations of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), particulate organic carbon (POC), dissolved inorganic nutrients (DIN), total suspended solids (TSS) and chlorophyll *a* (Chl *a*) concentration. Samples were further analysed according to the standard procedures of the Australian Institute of Marine Science (AIMS, Townsville, Australia; Devlin and Lourey 2000). Sediment samples were collected with 100 mL glass jars at 2 m depth and characteristics, such as grain size distribution and total organic carbon (TOC) and nitrogen (TON) content, were assessed for each sampling event. Seawater temperatures were obtained from AIMS long-term monitoring temperature records (http://eatlas.org.au/).

DNA extraction

Prior to extraction, the macroalgal biofilm was separated from the algal tissue by overnight incubation at 200 rpm in 10 mL 1 x PBS at 37℃. Coral fragments were defrosted on ice and the tissue was stripped from the skeleton with an airgun into 1 x PBS solution, homogenised for 1 min at 12,500 rpm with a tissue homogeniser, pelleted (10 min at 16,000 rcf) and snap frozen in liquid nitrogen prior to DNA extraction. DNA from seawater, sediment, sponge and macroalgal biofilms was extracted with the DNeasy PowerSoil kit (QIAGEN) and DNA of coral tissue and mucus samples was extracted using the DNeasy PowerBiofilm kit (QIAGEN) following the manufacturer's instructions. DNA extracts were stored at -80℃ until being sent for sequencing.

16S rRNA gene sequencing

DNA extracts were sent on dry ice to the Ramaciotti Centre for Genomics (Sydney, Australia) for sequencing. The bacterial 16S rRNA genes were sequenced using the 27F (Lane 1991) and 519R (Turner et al 1999) primer pairs on the Illumina MiSeq platform utilising a duel indexed 2 x 300 bp paired end approach. Further documentation outlining the standard operating procedures for generating and sequencing amplicons is available at https://data.bioplatforms.com/dataset/marine-microbes-methods.

Sequence analysis

Sequencing data were analysed as single nucleotide variants in a standardized platform alongside other Australian microbial biodiversity initiative samples (Bissett et al 2016, Brown et al 2018). In brief, forward and reverse reads were merged using FLASH (Magoc and Salzberg 2011). FASTA formatted sequences were extracted from FASTQ files and those < 400 bp in length or containing N's or homopolymer runs of > 8 bp were removed using MOTHUR (v1.34.1; Schloss et al 2009). USEARCH (64 bit v10.0.240) (Edgar 2010) package was used to de-replicate sequences and to order them by abundance. Sequences with < 4 representatives and Chimeras were removed. Quality-filtered sequences were mapped to chimera-free zero-radius operational taxonomic units (zOTUs) and a sample by read abundance table created. zOTUs were taxonomically classified with SILVA v132 (Yilmaz et al 2014) database using MOTHUR's implementation of the Wang classifier (Wang et al 2007) and a 60% Bayesian probability cut-off.

Chloroplast and mitochondria derived reads as well as singletons were removed from the dataset. Remaining data were rarefied to 3,600 reads per sample and transformed to relative abundances using the phyloseq package (McMurdie and Holmes 2013) in R (R Development Core Team 2008).

Habitat and host-specificity

Habitat and host-specificity of a microbiome was assessed by calculating the compositional similarities of all 381 samples with the Bray-Curtis Index and illustrating them in a Non-Metric Multidimensional Scaling (NMDS) plot using the phyloseq package (McMurdie and Holmes 2013). To confirm habitat and host-specificity, Permutational Multivariate Analysis of Variance (PERMANOVA) was applied using the adonis() function of the vegan package (Oksanen et al 2013) with 10,000 permutations.

Uniform response pattern

The microbiome similarity of replicates for sampling time points *versus* the microbiome similarity between sampling time points was compared by obtaining the Bray-Curtis Similarity for each habitat individually. The variation between the overall within and between time point replicates was tested with a Wilcoxon Rank-sum test in R (R Development Core Team 2008). The dispersion of the Bray-Cutis similarities within a sampling time point was calculated as the coefficient of variation (ratio of the standard deviation to the mean expressed as a percentage). The higher the coefficient of variation the higher the variability in the microbiome composition among replicates of a time point. Analysis of Similarity (ANOSIM; anosim() function of the vegan package; Oksanen et al 2013) based on Bray-Curtis similarities was used to further evaluate within and between time point similarities in the microbial communities.

Environmental sensitivities

Environmental metadata were z-score standardized (Clark-Carter 2014) and checked for collinearity using the Pearson correlation coefficient. Collinearity was assumed if correlation was > 0.7 or < -0.7 (Dormann et al 2013). Collinear variables were considered redundant and removed from the analysis.

zOTU relative abundance, environmental metadata (e.g., average seawater temperature, average hours of daylight, Chl *a*, POC, NPOC and TSS concentration), season (summer *versus* winter) and sampling date were used for Bray-Curtis distance-based redundancy analysis (dbRDA) using the phyloseq package (McMurdie and Holmes 2013). The significance of each response variable was confirmed with an Analysis of Variance (ANOVA) for the dbRDA (anova.cca() function in the vegan package; Oksanen et al 2013). Only significant (p-value < 0.05) response variables were kept in the model. The explanatory value (in %) of significant response variables (e.g., environmental parameters, season and sampling date) was assessed with a Variation Partitioning Analysis of the vegan package (Oksanen et al 2013).

Indicator value analysis

Indicator taxa were identified with the indicator value analysis (indicspecies package; De Cáceres and Legendre 2009) using the following thresholds: 1,000 permutations, minimum specificity (At) and minimum sensitivity (Bt) set to 70% and p-value ≤ 0.01.

Random forest machine learning

Random forest machine learning was performed with the caret (Kuhn 2008) and random forest package (Liaw and Wiener 2002) in R (R Development Core Team 2008). zOTUs with non-zero abundance values in at least 10% of the samples (n = 48) were preselected and z-score standardised prior to model training. Random Forest (with n_{trees} = 10,000) prediction error was measured with out-of-bag (OOB) error. Highest accuracy (lowest OOB estimated error rate) for classification was achieved with m_{try} = 100 zOTUS and for regression with m_{try} = 400 zOTUs. Importance of zOTUs was measured using the decrease in mean accuracy for classification and mean-squared error (% Inc. MSE) for regression.

Results

Samples were collected during a 16-month period (February 2016 - May 2017), at monthly (Magnetic Island - Geoffrey Bay) and periodic (Orpheus Island – Pioneer Bay and Channel) intervals (Appendix A Table S4.1). The bacterial 16S rRNA genes of 381 samples including seawater, sediment, sponge tissue (*Coscinoderma matthewsi* and *Amphimedon*

queenslandica), coral tissue and mucus (*Acropora tenuis* and *Acropora millepora*), and macroalgal surfaces (*Sargassum sp.*) were sequenced (Figure 4.1). In total 231,316 zero-radius operational taxonomic units (zOTUs) were identified based on 100 % sequence similarity (Brown et al 2018).

Coral reef microbiomes are habitat-specific

Habitat-specificity of coral reef microbes was assessed by comparing the similarities of microbial communities associated with seawater (n = 48), sediment (n = 48), *A. queenslandica* (n = 30), *C. matthewsi* (n = 42), *A. tenuis* (tissue n = 48, mucus n = 46*), A. millepora* (tissue n = 42, mucus n = 42) and *Sargassum sp.* (n = 35). Non-metric Multidimensional Scaling based on Bray-Curtis dissimilarities revealed a clear separation of the microbial communities from different reef habitats (Figure 4.1), and habitat-specificity was further confirmed with Permutational Multivariate Analysis of Variance (PERMANOVA, $p = 9.999 \times 10^{-5}$, Appendix A Table S4.2 - S4.3). Furthermore, alpha diversities (ANOVA, $F_{(8/372)} = 142$, $p = 2 \times 10^{-16}$) and zOTU richness (ANOVA, $F_{(8/372)} = 369$, $p = 2 \times 10^{-16}$) varied significantly between reef habitats (Appendix A Figure S4.1 and Appendix A Table S4.4-S4.6). Sediment harboured by far the most diverse (Shannon Index 7.4 ± 0.2 SD) bacterial community, although microbial diversity was also high in coral surface mucus (Shannon Index 5.1 ± 0.9 SD), macroalgal biofilms (Shannon Index 4.5 ± 1.4 SD), seawater (Shannon Index 4.4 ± 0.2 SD) and in the tissue of the sponge *C. matthewsi* (Shannon Index 4.4 ± 0.3 SD). Microbial diversity was lowest in coral tissue (Shannon Index 3.3 ± 0.8 SD) and in the sponge *A. queenslandica* (Shannon Index 2.7 ± 0.8 SD). These results suggest overall high habitat-specificity of free-living and host-associated microbial communities within coral reef ecosystems.

Uniform versus variable community assembly patterns

The uniformity *versus* variability of microbial community assembly patterns was explored through comparison of compositional similarity (Bray-Curtis index, 0 = dissimilar, 1 = identical) in samples collected monthly at Geoffrey Bay (Magnetic Island). The microbial communities of seawater (n = 30, Wilcoxon Rank-Sum test $p = 3.1 \times 10^{-7}$) and sediment (n = 30; Wilcoxon Rank-Sum test $p = 3 \times 10^{-5}$) had significantly higher similarities “within” than “between” sampling events (Figure 4.2a). This uniform response of the free-living microbial communities suggests that deterministic rather than stochastic processes drive their community assembly. For host-associated microbiomes, the overall response pattern varied between species. Microbial communities associated with the sponge *C. matthewsi* (n = 27; Wilcoxon Rank-Sum test, p = 0.0076), the coral *A. tenuis* (mucus n = 28, tissue n = 30; Wilcoxon Rank-Sum test, p = 0.0041 and p = 0.0096, respectively) and the macroalga *Sargassum sp.* (n = 30; Wilcoxon Rank-Sum test, p = 0.00013) followed the same trend as the free-living communities, with

significantly higher similarities "within" than "between" sampling events (Figure 4.2a). In contrast, the microbiome of the sponge *A. queenslandica* (n = 30; Wilcoxon Rank-Sum test, p = 0.23) and the coral *A. millepora* (mucus n = 24, tissue n = 24; Wilcoxon Rank-Sum test, p = 0.15 and p = 0.11 respectively) showed no significant difference in similarities "within" and "between" time points (Figure 4.2a). Analysis of the compositional similarity of sample replicates within each sampling time point indicated that the seawater microbial communities not only exhibit an overall higher similarity "within" replicates, but the high compositional similarity is conserved across all sampling events (Figure 4.2b). In contrast, host-associated microbial communities showed a generally lower compositional similarity and higher variation between sample replicates within each sampling time point (Figure 4.2b).

Trends in the temporal community assembly pattern of free-living, host tissue- and biofilm-associated microbial communities were analysed using Analysis of Similarity (ANOSIM) as a proxy to describe similarity patterns (R = 0 indicates equal similarity "within" and "between" time point replicates and R = 1 indicates higher "within" than "between" sampling time point similarities; Figure 4.2b and Appendix A Figure S4.2). Overall, free-living microbiomes had R values closer to 1 (seawater R = 0.9919 and sediment R = 0.7322), whereas host-associated microbiomes had R values closer to 0 (*A. queenslandica* R = 0.2927, *C. matthewsi* R = 0.3449, *A. tenuis* tissue R = 0.4547, *A. millepora* tissue R = 0.2151, *A. tenuis* mucus R = 0.4613, *A. millepora* mucus R = 0.3090 and *Sargassum sp.* biofilm R = 0.4440; Figure 4.2b and Appendix A Figure S4.2). These results suggest that free-living microbiomes (seawater and sediment) exhibit a uniform compositional succession, whereas host-associated microbiomes (coral, sponge and macroalgae) are more stochastic in their temporal community succession. The uniform temporal response of free-living microbiomes suggests a high diagnostic value of these microbial communities; hence seawater and sediment microbiomes should provide an accurate prediction of environmental variables.

Microbiomes in seawater (n = 48) and sediment (n = 48) were further tested for their compositional similarity between all three sampling sites (Geoffrey Bay, Pioneer Bay and Channel). The microbial community composition of sediment samples varied significantly between sampling sites (ANOSIM R = 0.9430, p = 0.001, Appendix A Figure S4.3a). The seawater microbiome, in contrast, showed high temporal variability (ANOSIM R = 0.9934, p = 0.001) and low spatial variability (ANOSIM R = 0.2343, p = 0.002; Appendix A Figure S4.3b). The high spatial variability of sediment microbiomes indicates that habitat characteristics rather than environmental fluctuations are the main drivers structuring community composition.

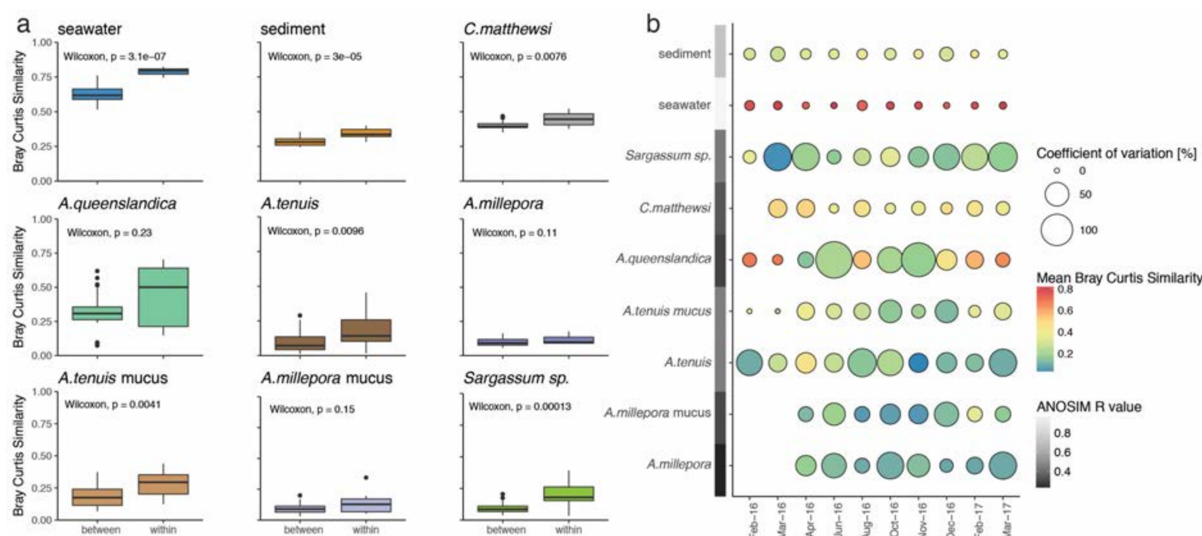

Figure 4.2. Compositional similarity of coral reef microbiomes over time a) Variations in the compositional similarity between and within sampling time points of various coral reef microbiomes collected at Geoffrey Bay (Magnetic Island). A higher similarity within time point replicates than between time point replicates suggests a uniform response of the microbial community to temporal variations. Similarities were calculated with Bray-Curtis Similarity Index (0 = no similarity, 1 = high similarity) and significances tested with Wilcoxon rank-sum test. b) The within sampling time point similarities of replicates (n=3) is indicated in colour and the dispersion (coefficient of variation - ratio of the standard deviation to the mean expressed as percentage) is displayed as size. Analysis of Similarity (ANOSIM) was further used as a proxy for the within and between time point variation. R-values of 1 indicate high similarity within sampling time points and high variability between sampling time points, whereas 0 indicates equal similarity within and between sampling time points.

Environmental sensitivity

Environmental sensitivity of the different microbiomes was assessed by comparing how much of the compositional variation was explained by sea surface temperature, light and water quality parameters (Appendices Figures S4.4 and S4.5). The compositional variability of the seawater microbiome (n=30) was significantly explained by sampling date, season (summer *versus* winter) and water quality parameters, such as average seawater temperature, average hours of daylight, total suspended solids (TSS), particulate organic carbon (POC), chlorophyll *a* (Chl *a*), and non-purgeable organic carbon (NPOC) concentration (permutational ANOVA for Bray Curtis distance based Redundancy Analysis (dbRDA); Figure 4.3a and Appendix A Table S4.7a-b). In total, these environmental parameters explained 56 % of the observed compositional variation in seawater (Variation Partitioning Analysis, Figure 4.3b, Appendix A Table S4.7). Season (summer *versus* winter) and sampling date solely explained 6 % and 4%, respectively (Variation Partitioning Analysis, Figure 4.3b). In comparison, sampling site significantly explained 24 % of the variation in sediment microbial communities (n = 48), which overlapped by 12% with the variation explained by sediment characteristics, such as particle size and total organic carbon (TOC) content (permutational ANOVA for dbRDA and Variation Partitioning Analysis; Appendix A Table S4.7b and S4.8). Water quality parameters and sea surface temperature explained only 3 % of the observed variability in the sediment microbiome (Variation Partitioning Analysis).

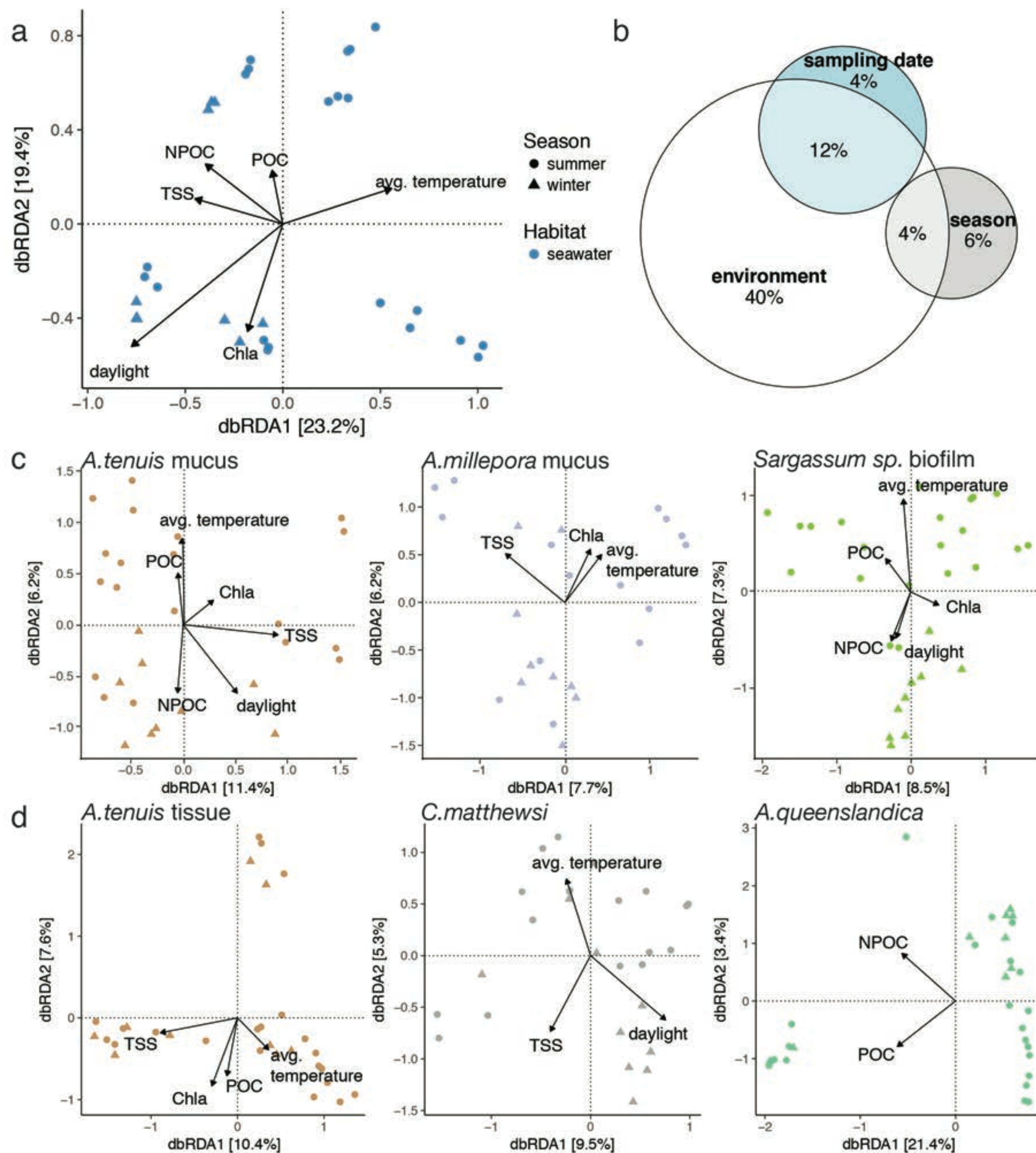

Figure 4.3. Coral reef microbiome sensitivity to environmental parameters. Bray-Curtis distance-based RDA (dbRDA) was used to evaluate the effect of environmental fluctuations on the microbial community composition of various coral reef habitats/hosts. The total variance (in percent) explained by each axis is indicated in parentheses. a) Environmental factors (average temperature, daylight, TSS, NPOC, Chl *a* and POC) significantly explained the observed compositional variation in the seawater-associated microbial community (permutational ANOVA for dbRDA). b) Variation partitioning shows that environmental parameters (average temperature, daylight, TSS, NPOC, Chl *a* and POC) rather than season and/or sampling date explain observed community composition structures in the seawater microbiome. c) Coral mucus and algae biofilm as well as d) coral and sponge tissue microbial communities were significantly influenced by environmental factors; however, environmental parameters only explain on average 11% of the observed community variation (Appendix A Table S4.7).

Host-associated microbiomes varied substantially in their response to environmental parameters (permutational ANOVA for dbRDA and Variation Partitioning Analysis, Figure 4.3b-c, Appendix A Table S4.7c-i and S4.8). On average, 11 % of the observed community variations in host-associated microbiomes were explained by the environment (Variation Partitioning Analysis), which is five-times less than what we found for the seawater associated

microbial community (Appendix A Table S4.8). This suggests that compositional variations of the seawater microbiome are more likely to reflect environmental changes. Host-associated microbiomes, are comparatively stable to changes in environmental factors.

Predictability of environmental metadata

Due to the seawater microbiomes uniform temporal pattern and high sensitivity to changing environmental parameters, the ability to infer environmental state based on microbial community data was tested using an Indicator Value analysis (De Cáceres and Legendre 2009) and a Random Forest machine learning approach. In total, 110 zOTUs were identified as significant indicators for temperature (Indicator Value $p < 0.01$). Microbial zOTU assemblages that were indicative of high, low and average seawater temperatures (classification based on their variation around observed annual averages) were present throughout the sampling period. However, higher relative abundances and lower variation (as calculated by coefficient of variation) were evident at certain time points (Figure 4.4a). Furthermore, we were able to identify microbial indicator taxa for high and low Chl *a*, TSS and POC levels (Appendix A Figure S4.6). Indicators for low and high seawater temperatures were identified in the bacterial phyla *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria* and *Planctomycetes* (Figure 4.4b). High temperatures were indicated by an increase of zOTUs belonging to the bacterial family *Rhodobacteraceae* and the presence of *Cryomorphaceae*, *Synechococcaeae*, *Vibrio* and *Flavobacterium* (Figure 4.4b). In contrast, the occurrence of zOTUS belonging to the family *Pelagibacteriaceae* and the genus *Prochlorococcus* were indicative for low seawater temperatures. The phyla *Proteobacteria*, *Bacteroidetes* and

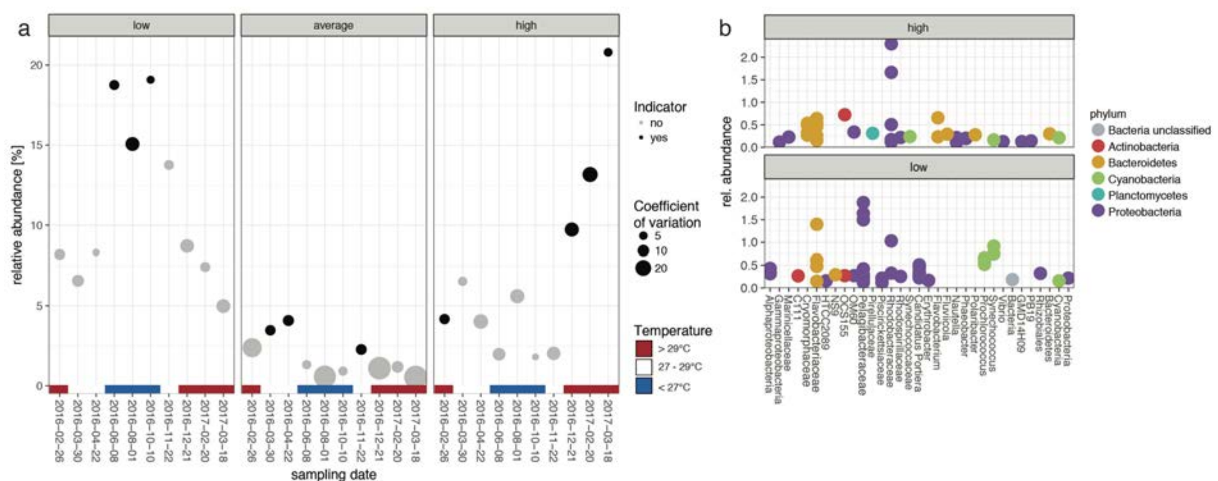

Figure 4.4. Microbial indicator taxa for seawater temperature fluctuations. Seawater temperatures were z-score standardised and, based on the variation around their mean, classified into low (< -0.5), average (-0.5 – 0.5) and high (> 0.5) temperature groups. Indicator zOTUs were identified with the Indicator Value analysis (IndVal). a) The average relative abundance of the sum of low, average and high temperature indicators is represented for each sampling time point. Significant indicator zOTUs assemblages ($p < 0.01$) for the respective temperature group are indicated in black and size represents the coefficient of variation. Colour gradient further represents the seawater temperature at the given sampling time points. b) Relative abundances and taxonomic affiliation of zOTUs identified to be significant ($p < 0.01$) indicators for high and low seawater temperatures. Each dot represents a unique zOTU.

Cyanobacteria had the greatest number of indicator zOTUs for temperature and other water quality parameters (Appendix A Figure S4.6). *Flavobacteriaceae*-affiliated zOTUs were significant indicators for temperature, Chl *a*, TSS and POC. *Halomonadaceae* significantly associated with high Chl *a* and TSS and zOTUs belonging to the phylum *Verrucomicrobia* were significant indicators for high TSS levels.

The diagnostic value of the seawater microbiome (n = 48) was further evaluated by applying a Random Forest machine learning classification and regression analysis with 1,213 zOTUs preselected based on a non-zero abundance threshold in at least 10 % of the samples (n = 48). The seawater microbiome enabled the prediction of seawater temperature classes (low, average, high) with 92 % accuracy (Kappa = 88 %, Figure 4.5a-b and Appendix A Figure S4.7). Highest accuracy (lowest Out of Bag (OOB) estimated error rate) was achieved with m_{try} = 100 zOTUS. Random Forest regression of the seawater microbiome predicted temperature values (R^2 = 0.67, RMSE = 0.5) (Figure 4.5c-d and Appendix A Figure S4.8) with the highest accuracy (lowest OOB estimated error rate) when m_{try} = 400 zOTUs. The effectiveness of zOTUs in reducing uncertainty and variance (also referred to as 'feature importance') within the machine learning algorithm was measured by the decrease in mean accuracy for classification and mean-squared error (% Inc. MSE) for regression. The most important zOTUs belong to the bacterial taxa *Flavobacteriaceae*, *Pelagibacteraceae*, *Cyanobacteria*, *Rhodobacteraceae*, *Synechococcaceae* and *Pirrelulaceae*. These results demonstrate that the microbial community associated with coral reef seawater allows for the accurate prediction of fluctuations in sea surface temperature and water quality parameters.

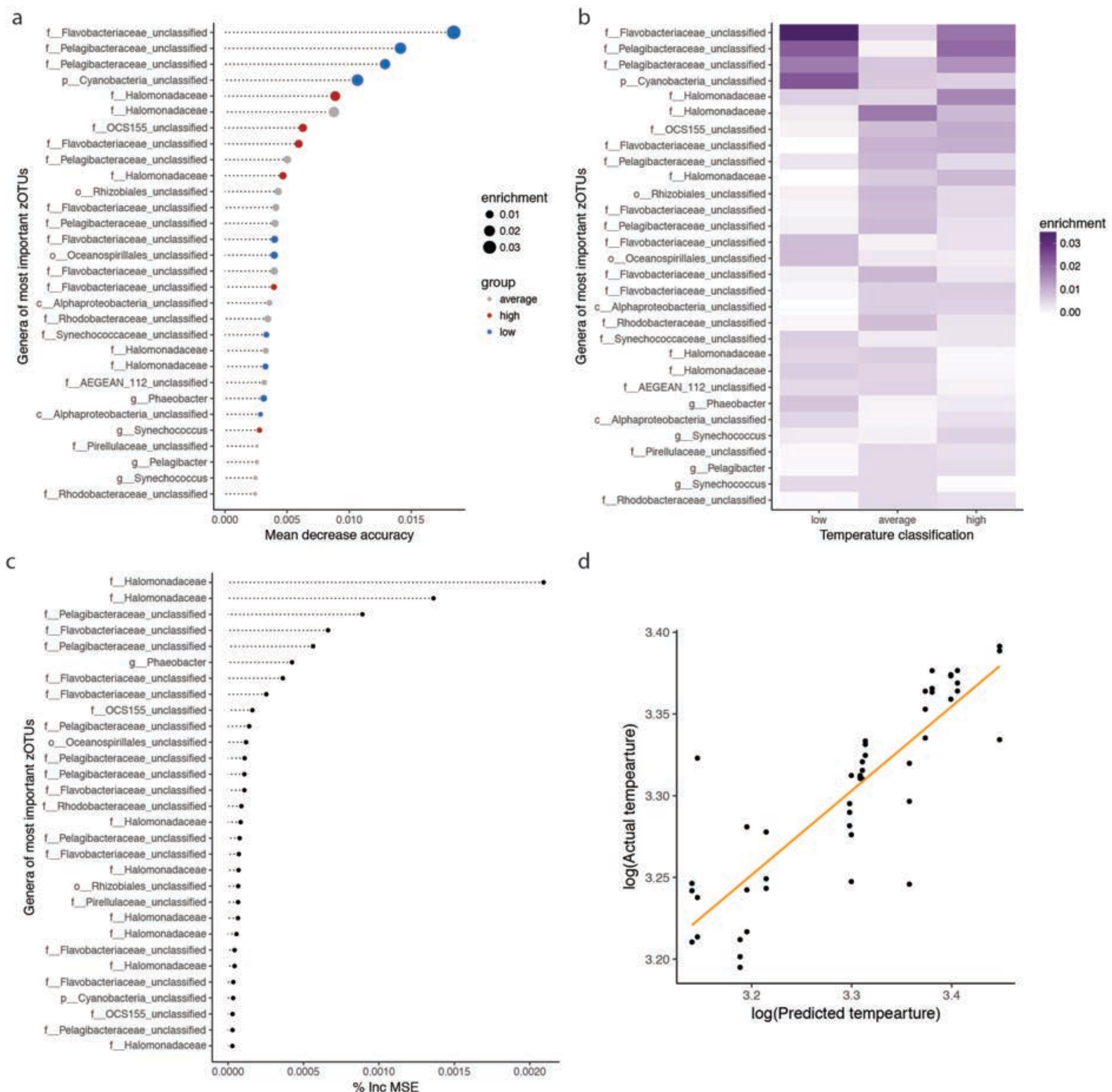

Figure 4.5. Random Forest machine learning a) The 30 most important zOTUs reducing the uncertainty in the prediction of seawater temperature classes (low, average, high) based on their mean decrease in accuracy and b) their enrichment in the temperature classes. c) The 30 most important zOTUs reducing the variance (mean squared error (% Inc MSE)) in regression based prediction of seawater temperatures. d) Predicted seawater temperature values *versus* actual seawater temperature values based on Random Forest regression.

Discussion

Sensitive and rapidly responding markers of coral ecosystem stress are needed to underpin effective management and restoration strategies. In this study, we used a range of statistical tests and machine learning approaches across multiple free-living and host-associated reef microbiomes to assess their diagnostic value as sensitive indicators of environmental state. Our results show that the microbial community in reef seawater has the highest diagnostic

value when compared to other free-living (e.g. sediment) and host-associated microbiomes (e.g., coral, sponge and macroalgae). Our conclusion is based on the microbiome's 1) habitat-specificity, 2) uniformity of its community assembly, 3) sensitivity towards environmental fluctuations and 4) accuracy to predict environmental parameters. This assessment of the diagnostic capacity of various free-living and host-associated coral reef microbiomes to extrapolate environmental variations provides crucial information for ecosystem management initiatives aimed at incorporating microbial monitoring.

In general, high habitat-specificity was observed across free-living and host-associated microbiomes, confirming previous reports on the compositional variability of microbial communities between coral reef habitats (Tout et al 2014), host species (Carlos et al 2013, Glasl et al 2018b, Rohwer et al 2002, Webster and Thomas 2016) and even between host compartments (Sweet et al 2011). High compositional divergence of microbial communities across different reef habitats can be due to the variation of available resources and/or biotic interactions (Martiny et al 2015). High habitat-specificity contributes to the overall high diversity and complexity across different microbial communities on coral reefs, highlighting the importance of holistic studies that focus on microbial interactions across the benthic-pelagic realm.

Bacterial community structure associated with water and sediment is thought to be primarily governed by deterministic processes (Wang et al 2013). Our results are consistent with this, showing uniform community assembly patterns within time point replicates. In contrast, host-associated microbiomes displayed little compositional similarity within a sampling time point, suggesting a non-uniform temporal response. Host-associated microbiomes were also only marginally affected by environmental parameters, indicating that their community assembly patterns are variable between conspecific individuals (Wang et al 2013). A higher variability in community assembly can lead to increased community heterogeneity, also referred to as dispersion, which has been described as a common characteristic of host-associated microbiomes (Casey et al 2015, Glasl et al 2016, Zaneveld et al 2016, Zaneveld et al 2017). Furthermore, lower microbial compositional similarities amongst replicates may be driven by increased niche space (e.g. host compartments; Sweet et al 2011) and host genotype effects (e.g. host genetics; Glasl et al 2018b). Collectively, our results show that free-living microbial communities have a higher potential to infer environmental parameters (such as standard measures in environmental monitoring programs) than host-associated microbial communities due to their higher uniformity and environmental sensitivity. Importantly however, previous metaproteomic research on reef sponges has shown that while microbial community composition can appear stable when seawater temperatures increase, disruption to nutritional interdependence and molecular

interactions (such as reduced expression of transporters involved in the uptake of sugars, peptides and other substrates) actually occurs prior to detectable changes in community structure (Fan et al 2013). Hence, considering the importance of microbes to reef invertebrate health, more sensitive transcriptomic / proteomic approaches may still be warranted for sensitive detection of microbial responses to environmental perturbations.

The diagnostic potential of microbial communities, especially in combination with machine learning approaches, has gained momentum across multiple research fields, including disease identification by characterisation of the human gut-microbiome (Duvallet et al 2017), evaluation of the environment and host genetics on the human microbiome (Rothschild et al 2018), prediction of hydrological functions in riverine ecosystems (Good et al 2018) and assessment of macroecological patterns in soil samples (Ramirez et al 2018). This development of microbial-based diagnostics is largely due to availability of high-throughput sequencing of the 16S rRNA gene and streamlined analytical pipelines that facilitate rapid assessment of microbial community composition (Schuster 2008, Waldor et al 2015). In addition to its utility for inferring environmental fluctuations, the seawater microbiome possesses numerous characteristics desirable for environmental monitoring programs: i) non-destructive collection and simple processing methods facilitate large-scale collections alongside existing programs that sample water quality measurements, ii) high fractional contribution of abundant microbes minimises the impacts of sequencing biases (Appendix A Figure S4.9) and iii) sampling is conducive to future automated, high throughput analyses such as in-line flow cytometry on vessels and real-time DNA/RNA sequencing for community characterisation.

Incorporation of seawater microbial community data into coral reef monitoring approaches should enhance our ability to describe environmental conditions and changes more holistically. For example, temperature fluctuations drive structural variations in seawater microbial communities (Roik et al 2016, Sunagawa et al 2015), and elevated seawater temperatures on coral reefs are highly correlated with coral bleaching (Brown 1997, Hughes et al 2017a). The inclusion of microbial community data alongside water quality parameters could therefore improve our ability to predict the likelihood of ecosystem stress. For instance, our sample sites, located in the central sector of the GBR, were not affected by the 2016 bleaching that primarily affected the northern sector (Hughes et al 2017b), however they were impacted by the 2017 bleaching event (ARC Centre of Excellence 2017). In the months prior to bleaching (late December 2016 till March 2017) we observed two to four times higher relative abundances of high temperature indicator assemblages than when compared to the equivalent period at the beginning of 2016 (Figure 4.4a), where no bleaching was observed. Interestingly, high temperature indicator assemblages included putative coral pathogens (e.g.

Vibrio) and opportunistic bacteria (e.g., *Rhodobacteraceae*, *Verrucomicrobia* and *Flavobacterium*). Coral pathogens, such as *Vibrio corallilyticus* increase their efficiency and motility behaviours with rising seawater temperatures (Garren et al 2014, Garren et al 2016, Tout et al 2015), and the higher abundance of these microbes may explain the increased prevalence of coral disease post bleaching (Muller et al 2008, Mao-Jones et al 2010). Hence, microbial monitoring could help inform managers about impending disease outbreaks.

Conclusion

Our study provides the first holistic microbial baseline spanning multiple free-living and host-associated microbiomes for selected GBR sites. Results suggest that there is realistic scope to enhance long-term reef monitoring initiatives by incorporating seawater microbiome observations for assessments of environmental change over space and time, especially for rapid and sensitive identification of early signs of declining ecosystem health. The establishment of microbial observatories (Buttigieg et al 2018) and DNA biobanks for long-term biomonitoring (Jarman et al 2018) will be paramount to successfully inferring ecosystem state and / or perturbations from microbial communities. We therefore recommend timely integration of microbial sampling into current coral reef monitoring initiatives. Further refinement of the sampling and data analysis techniques should focus on selection and validation of additional indicator taxa as well as assessment of ecologically important microbial functions. A further consideration is to explore which monitoring objectives would benefit most from assessments of microbial communities. For example, it is likely that the rapid response time of microbial indicators makes them better suited to early-warning, impact or compliance monitoring programs than to monitoring of slower, long-term changes.

Chapter 5

EXPLORING THE DIVERSITY-STABILITY PARADIGM USING SPONGE MICROBIAL COMMUNITIES

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Abstract

A key concept in theoretical ecology is the positive correlation between biodiversity and ecosystem stability. When applying this diversity-stability concept to host-associated microbiomes, the following questions emerge: 1) Does microbial diversity influence the stability of microbiomes upon environmental fluctuations? 2) Do hosts that harbor high *versus* low microbial diversity differ in their stress response? To test the diversity-stability concept in host-associated microbiomes, we exposed six marine sponge species with varying levels of microbial diversity to non-lethal salinity disturbances and followed their microbial composition over time using 16S rRNA gene amplicon sequencing. No signs of sponge stress were evident following salinity amendment and microbiomes exhibited compositional resistance irrespective of their microbial diversity. Compositional stability of the sponge microbiome manifests itself at distinct host taxonomic and host microbial diversity groups, with 1) stable host genotype-specific microbiomes at oligotype-level; 2) stable host species-specific microbiomes at genus-level; and 3) stable and specific microbiomes at phylum-level for hosts with high *versus* low microbial diversity. The resistance of sponge microbiomes together with the overall stability of sponge holobionts upon salinity fluctuations suggest that the stability-diversity concept does not appear to hold for sponge microbiomes and provides further evidence for the widely recognized environmental tolerance of sponges.

Introduction

Marine invertebrates establish relationships with a wide diversity of microorganisms that undertake fundamental roles in host nutrition, waste-product removal, host immunity, pathogen defence and host development (Bourne et al 2016, McFall-Ngai et al 2013, Webster and Thomas 2016). The ecological unit comprised of the animal host and its associated microbes is often referred to as a holobiont (Rohwer et al 2002, Rosenberg and Zilber-Rosenberg 2011), where the associated microbes are not a random aggregation of environmental microorganisms but rather a selected consortium, critical to the well-being of the host (Bordenstein and Theis 2015, McFall-Ngai et al 2013). Disturbances or changes in the environment can destabilize the microbiome, often with adverse consequences for host health (Glasl et al 2016, Thurber et al 2014, Zaneveld et al 2016).

The application of concepts developed for the field of community ecology can be useful to better understand environmental drivers of microbial community dynamics (Christian et al 2015, Costello et al 2012). Similar to ecological communities (Allison and Martiny 2008), microbial communities can respond to disturbance events in different ways (Shade et al 2012).

For example, a microbiome can be entirely resistant to a stressor and hence no change in the community composition occurs (e.g., Luter et al 2014, Pineda et al 2016, Ziegler et al 2017). Alternatively, resilient microbial communities may shift immediately following the disturbance event but return to their original composition once the stressor(s) has been removed (e.g. Glasl et al 2016). However, if the shift is too dramatic or the original composition cannot be restored, the holobiont homeostasis can collapse which is often associated with disease and/or host mortality (e.g., Blanquer et al 2016, Fan et al 2013, Glasl et al 2016, Pineda et al 2016). The type of response a microbiome will exhibit upon disturbance is difficult to predict and likely depends on the nature of host-microbe association (facultative *versus* obligate), plus the strength and/or duration of the disturbance (Dini-Andreote et al 2015). Another potential factor may be the diversity (defined as richness and evenness) of a microbiome. Increased biodiversity, for example, has been postulated to increase the stability of an ecosystem (McCann 2000). For hosts associated with highly diverse microbiomes, these associations may provide greater functional repertoires and functional redundancies compared to animals that host less diverse microbiomes.

The association between sponges and their microorganisms represents one of the most evolutionarily ancient examples of symbiosis in multicellular life (Hentschel et al 2012, Webster and Thomas 2016). The diversity of microorganisms within sponges varies considerably amongst species (Reveillaud et al 2014, Webster et al 2010) and between sponges that host high (high microbial abundance; HMA) or low (low microbial abundance; LMA) microbial abundance (Hentschel et al 2003, Moitinho-Silva et al 2017). In general, microbial composition also differs between HMA and LMA species, with LMA sponges being dominated by *Proteobacteria* and *Cyanobacteria* (Erwin et al 2015, Giles et al 2013, Hentschel et al 2006) and HMA sponges being dominated by the phyla *Chloroflexi*, *Acidobacteria*, *Actinobacteria* and PAUC34f (Moitinho-Silva et al 2017). HMA and LMA sponge species are also thought to differ in their functional gene content (Bayer et al 2014), pumping rates (Weisz et al 2008), and their cycling of carbon and nitrogen compounds (Ribes et al 2012). Although notable similarities in microbiome stability over seasonal scales has been detected across the HMA-LMA dichotomy (Erwin et al 2015), how microbial diversity and abundance affects sponge microbiome stability upon acute environmental fluctuations has not yet been defined.

This study investigates how the diversity of the sponge microbiome influences community stability upon acute salinity fluctuations (ranging from 36 psu to 25 psu) under controlled experimental conditions (Figure 5.1). The simulated fluctuation mimics natural salinity levels experienced by reef organisms after major flooding events (Devlin and Schaffelke 2009, VanWoesik et al 1995), and therefore provides further insights into the environmental tolerance (ability to live within a certain range of abiotic factors) of sponge

holobionts to short-term salinity stress. Stability was investigated for six marine sponge species (*Amphimedon queenslandica*, *Ianthella basta* and *Stylissa flabelliformis* as representatives of low microbial diversity species; and *Coscinoderma matthewsi, Cymbastela coralliophila* and *Ircinia ramosa* as representatives of high microbial diversity species) using high taxonomic resolution based on Amplicon Sequence Variants (ASV) (Callahan et al 2017), facilitating detection of fine-scale variations in microbiome composition.

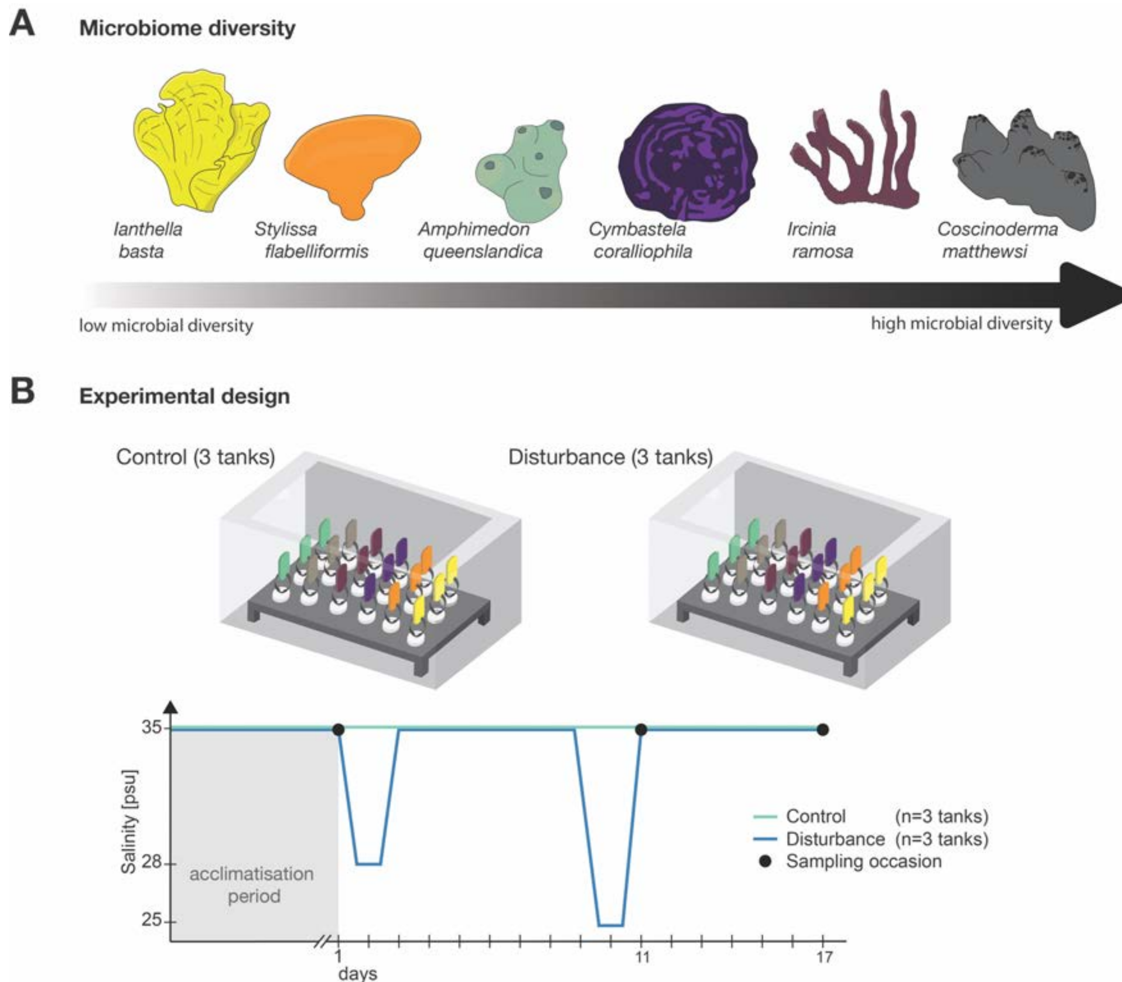

Figure 5.1. Diversity of sponge microbiomes and experimental setup to test microbiome stability. A) Sponge microbiomes vary substantially in their diversity, ranging from very low (Shannon index of approximately 1.3) to very high (Shannon index of approximately 4.9) microbial diversity. B) In total, six sponge genotypes per species were collected and each genotype was fragmented into three equally sized clones. Clones of each genotype were placed into the same experimental tanks to enable sub-sampling over time. The experimental design comprised three control tanks and three disturbance tanks, with each tank containing 18 sponge clones in total. Sponge clones were acclimatized to experimental conditions for one week and then one clone / genotype was sampled across all tanks immediately prior to the first disturbance event. One additional clone / genotype was sampled for each experimental tank 24h and 168h after the second pulse disturbance. Sponges in disturbance tanks experienced two consecutive salinity drops (28 psu and 25 psu, respectively), whereas sponges in control tanks were maintained at stable ambient salinity (35 psu) over the duration of the experiment.

Materials and Methods

Experimental setup

Great Barrier Reef (GBR) sponge species (n=6) associated with previously documented low and high diversity microbial communities (Spor et al 2011, Thomas et al 2016) were selected for the study and included: *Amphimedon queenslandica*, *Ianthella basta* and *Stylissa flabelliformis* as representatives of low microbial diversity species; and *Coscinoderma matthewsi, Cymbastela coralliophila* and *Ircinia ramosa* as representatives of high microbial diversity species. In total, six individuals of each sponge species were collected from Magnetic Island (*C. matthewsi* and *A. queenslandica*, Australia) and Davies Reef (*C. coralliophila*, *I. basta*, *I. ramosa* and *S. flabelliformis*; Australia) in February 2017. Samples were collected under the permits G12/35236.1 and G16/38348.1 granted by the Great Barrier Reef Marine Park Authority to the Australian Institute of Marine Science. All sponges were immediately transferred to the National SeaSimulator at the Australian Institute of Marine Science (Townsville, Australia), where sponges were kept in flow-through outdoor tanks under natural lighting. Within two days of collection, each sponge was fragmented into three equally sized clones and placed into indoor flow-through tanks for two weeks to allow tissue healing. Sponge clones were subsequently transferred to experimental tanks and left to acclimatize for seven days. Each experimental tank harboured six sponge species, each represented by three clones of the same individual (in total 6 x 3 sponge clones per tank; see Figure 5.1).

The experimental setup comprised three control tanks and three pulse salinity disturbance tanks. All tanks were kept at stable temperature (27.5°C ± 0.04°C), light (80 mol photons m^{-2} s^{-1}) and flow (8 m s^{-1}) conditions throughout the experiment. While control tanks were kept at stable ambient salinity (34.77 psu ± 1.05 psu), disturbance tanks were exposed to two consecutive pulse salinity drops on the second (day 2) and tenth day (day 10) to 28 psu and 25 psu, respectively (Figure 5.1). Each pulse lasted for a total of nine hours with the intensity and duration of the simulated salinity fluctuations based on previously documented salinity fluctuations on the GBR (e.g., Devlin and Schaffelke 2009, Kline et al 2015, VanWoesik et al 1995). Samples were collected before the disturbance (day 1), directly after the second low-salinity pulse event (day 11) and one week after the pulse event to assess recovery (day 17). On each sampling occasion one clone of each individual sponge was removed from the tanks with sterile tweezers, photographed, rinsed with 0.2 µm filtered seawater to remove loosely attached microbes from the surface and cut into small fragments. Randomly selected subsamples containing pinacoderm and mesohyl were placed into two 2 ml cryogenic vials (Corning), snap frozen in liquid nitrogen and stored at -80°C until further processing.

Pigment analysis

The concentration of sponge photopigments was analysed following the method described by Pineda et al (2016). Briefly, sponge samples were defrosted, wet weight of each sample was recorded (approximately 0.2 g) and samples were transferred into clean PowerBead tubes (MoBio Power Plant kit) containing four stainless steel beads per vial. To each tube 1 mLof 95% EtOH was added, and tissue was bead beaten for 3 x 40 s at 5 m s^{-1} and centrifuged for 30 s at 10,000 rcf. The supernatant was added in triplicate into 96-well plates and absorbance was measured at 470 nm, 632 nm, 649 nm, 665 nm, 696 nm and 750 nm on a Bio-Tek Power Wave Microplate Scanning Spectrophotometer. Blank-corrected absorbance readings were used to calculate chlorophyll *a*, *b*, *c*, *d*, total chlorophyll and total carotenoids (Appendix B Equation S5.1). Pigment concentration was normalized to sponge wet weight.

DNA extraction and sequencing

DNA was extracted from all sponge samples using the MoBio Power Soil kit following the manufacturer's instructions, including one bead beating step of 40 s at 4 m s^{-1}. DNA extracts were stored at -80°C until shipment on dry ice to Ramaciotti Centre (University of New south Wales, Australia) for sequencing. The V1-V3 region of the 16S rRNA gene was amplified using primers 27F (Lane 1991) and 519R (Turner et al 1999) and libraries were prepared with the Illumina TruSeq preparation protocol, followed by Illumina MiSeq 2 x 300 bp sequencing.

Sequence analysis

Demultiplexed paired end reads were analysed using QIIME2 (Version 2017.9.0; https://qiime2.org). Based on quality plots, forward and reverse reads were truncated at their 3' end at the 296 and 257 sequencing positions, respectively. Samples were individually checked for chimeras and chimeric sequences were removed from the dataset using DADA2 (Callahan et al 2016). Sequences were grouped into features based on 100 % sequence similarity, subsequently referred to as ASV (amplicon sequence variants), using DADA2 (Callahan et al 2016). Multiple *de novo* sequence alignments of the representative sequences was performed using MAFFT (Katoh et al 2002). Non-conserved and highly gapped columns from the alignment were removed using default settings of the mask option in QIIME2. Unrooted and rooted trees were generated using FastTree for analysis of phylogenetic diversity. For taxonomic assignment, a Naïve-Bayes classifier was trained on the SILVA v123 99 % Operational Taxonomic Units, where reference sequences only included the V1-V2 regions (27F/519R primer pair) of the 16S rRNA genes. The trained classifier was applied to the representative sequences to assign taxonomy. Chloroplast and mitochondria derived sequence reads and singletons were removed from the dataset and the feature table was rarefied to an even sequencing depth of 5,976 sequencing reads, representing 21.41 % of the

total sequences post quality control.

Statistical analyses were performed in R (R Development Core Team 2008). Multivariate statistical approaches including Analysis of Similarity (ANOSIM, 'vegan package'; Oksanen et al 2013), Permutation Multivariate Analysis of Variance (PERMANOVA, 'vegan package'; Oksanen et al 2013), Multivariate Homogeneity of Group Dispersion/Variance ('vegan package'; Oksanen et al 2013) and Non-metric multidimensional scaling (NMDS, 'phyloseq package'; McMurdie and Holmes 2013) were based on Bray Curtis dissimilarities. Graphs were created in R using ggplot2 (Wickham 2009) and phyloseq packages (McMurdie and Holmes 2013). The alluvial diagram was generated in RAWGraph (Mauri et al 2017).

Demultiplexed sequences and metadata are available from the Sequence Read Archives (SRA) under accession number SRP131926.

Results

Host health and photopigment composition

Sponges were not visibly stressed following salinity amendment as determined using the previously described stress proxies of mucus production, tissue regression and tissue necrosis (Pineda et al 2016). Photopigment concentrations (chlorophyll *a*, *b*, *c*, *d*, total chlorophyll and total carotenoids) were evaluated for each species as an additional proxy of host health (Appendices Material, Figure S5.1). Photopigment concentrations varied significantly between host species (ANOVA, $F_{(5/630)} = 8.145$, $p = 1.84 \times 10^{-7}$). *S. flabelliformis* had the highest total carotenoid concentration (150.57 µg g^{-1} ± 48.51) followed by *I. basta* (41.41 µg g^{-1} ± 9.48). Chlorophyll *a* concentration was highest in the two photosynthetic species *I. ramosa* and *C. coralliophila* ranging from 100.63 µg g^{-1} ± 37.60 to 97.20 µg g^{-1} ± 33.79 respectively. Neither time nor treatment had an effect on the photopigment composition within each host species (PERMANOVA, $p > 0.05$, Appendices Table S5.1).

Microbiome diversity and richness

In total, 7,077,372 Illumina sequence reads were obtained (ranging from 5,976 to 57,917 in the different samples), of which 3,185,811 reads remained after quality filtering. Overall, 6,896 ASV were identified based on single nucleotide variations in the sequence reads. The highest richness was observed in *A. queenslandica* (297 ASVs ± 94), while *I. basta* was associated with the lowest microbial richness (66 ASVs ± 62; Appendix B Table S5.2). Alpha diversities based on Shannon Index varied significantly between sponge species (ANOVA, $F_{(5/72)} = 85.356$, $p = 2 \times 10^{-16}$, Appendix B Table S5.3; Figure 5.2). *C. matthewsi* was associated with the highest alpha diversity (4.69 ± 0.18), followed by *I. ramosa* (3.69 ± 0.10), *C. coralliophila*

(3.14 ± 0.23), *A. queenslandica* (2.97 ± 0.71) and *S. flabelliformis* (2.61 ± 0.68). *I. basta* associated microbiomes had the lowest microbial diversity (1.52 ± 0.54). Sponges from the different treatment groups (control *versus* disturbance) had similar diversity values, indicating acute salinity disturbance had no influence on microbiome richness or evenness within each sponge species (Figure 5.2).

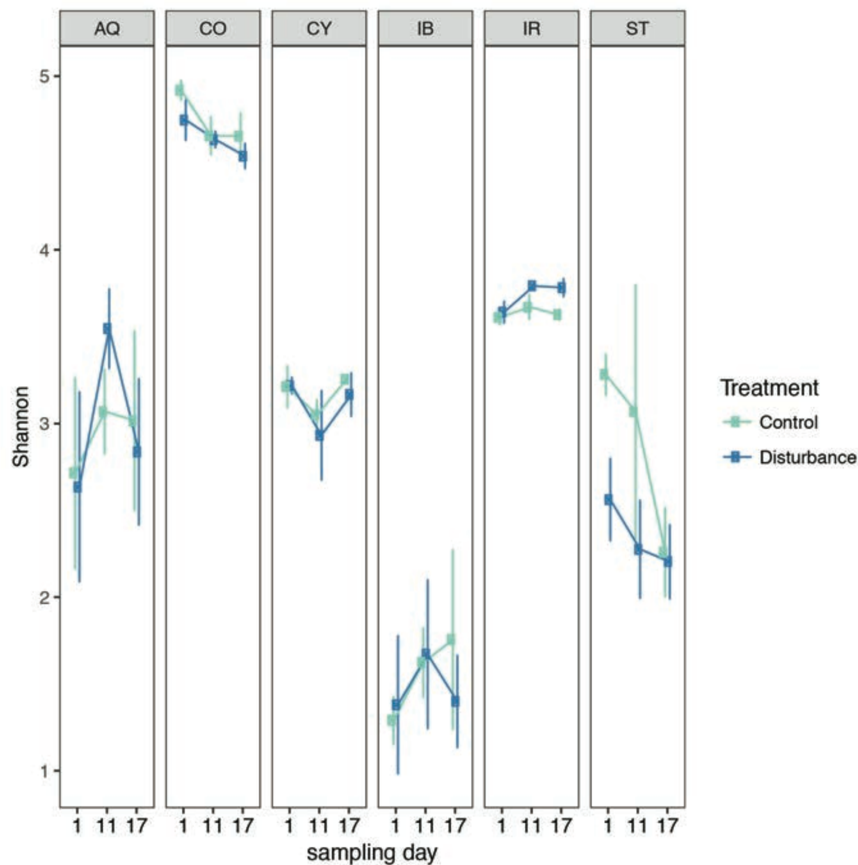

Figure 5.2. Variation in Shannon diversity (mean ± SD) in each sponge species across treatments and sampling times. *Amphimedon queenslandica* (AQ), *Coscinoderma matthewsi* (CO), *Cymbastela coralliophila* (CY), *Ianthella basta* (IB), *Ircinia ramosa* (IR) and *Stylissa flabelliformis* (ST).

Compositional stability of sponge microbiomes after salinity fluctuations

The stability of the sponge microbiome upon two consecutive pulses of reduced salinity was compared across HMA and LMA species. Each sponge species was associated with a distinct microbial community (ANOSIM, p = 0.001, R = 0.9793) and microbiomes of both treatment groups (control *versus* disturbance) were highly similar within each sponge species (ANOSIM p = 0.027, R = -0.0070; Figure 5.3). Multivariate dispersion (heterogeneity of a community based on distances of samples to their group centroid) of microbial assemblages varied significantly between sponge species (ANOVA, $F_{(11/96)}$ = 42.383, p = 2.2 x 10^{-16}; Figure 5.4),

however, treatment had no effect on the dispersion of the sponge microbiome (TukeyHSD p > 0.05, Appendix B Table S5.4). Microbial community composition in each sponge species also remained stable over time within each treatment group (adonis2, host and treatment group as blocking factor, 10,000 permutations, p = 0.9989, Appendix B Table S5.5). However, host genotype had a significant effect on microbial composition for all sponge species, with a higher similarity between samples originating from the same genotype than between samples originating from different conspecific genotypes (ANOSIM, p = 0.001, R = 0.9427). Furthermore, the microbiome composition varied significantly between sponge individuals (genotypes) of the same species (adonis2, host species as blocking factor, 10,000 permutations, p < 0.001, Appendix B Table S5.6).

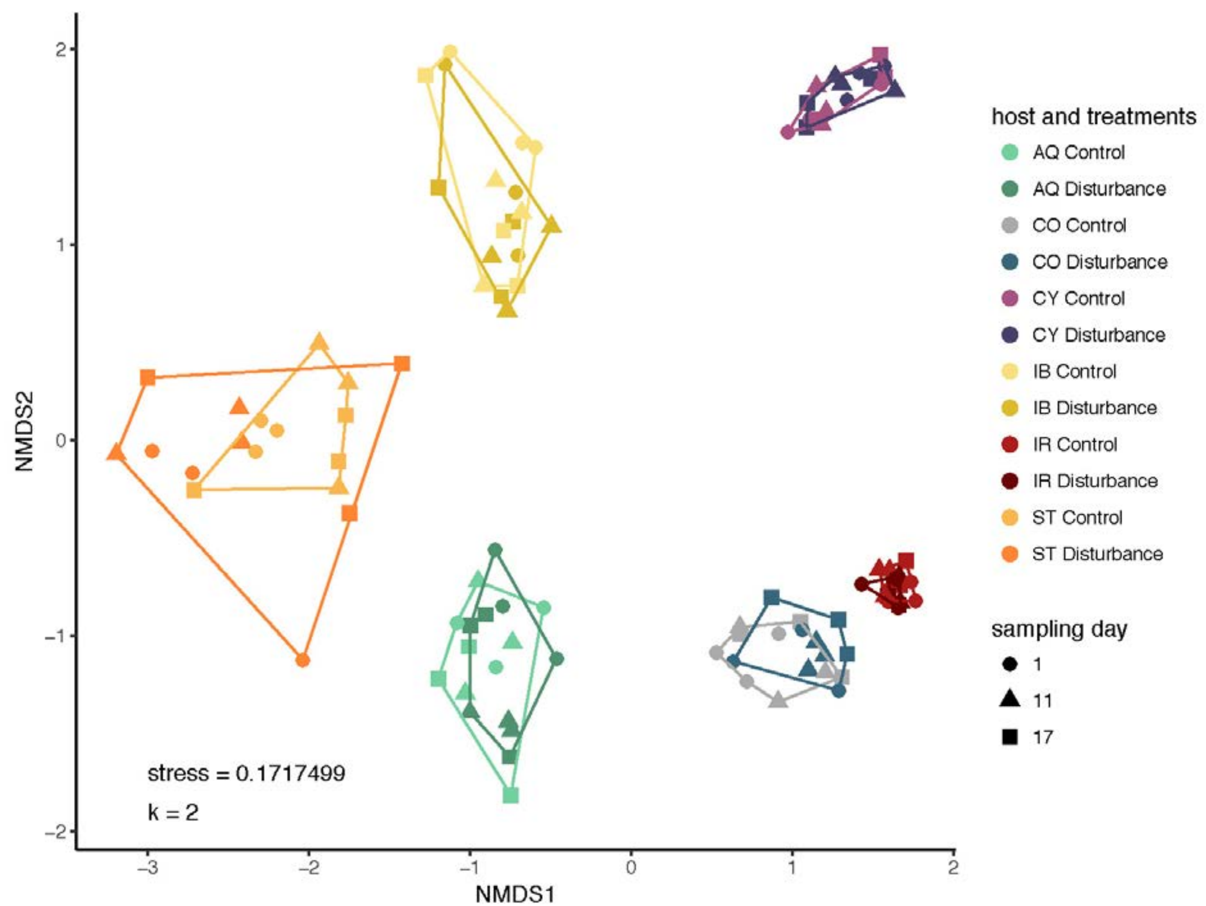

Figure 5.3. Non-metric multidimensional scaling plot displaying similarities in the microbiomes of the six sponge species under both treatment conditions (control and disturbance). Microbiomes show high host-species specificity and high temporal stability even after exposure to a non-lethal salinity stress. Abbreviation of the host species as indicated: *Amphimedon queenslandica* (AQ), *Coscinoderma matthewsi* (CO), *Cymbastela coralliophila* (CY), *Ianthella basta* (IB), *Ircinia ramosa* (IR) and *Stylissa flabelliformis* (ST).

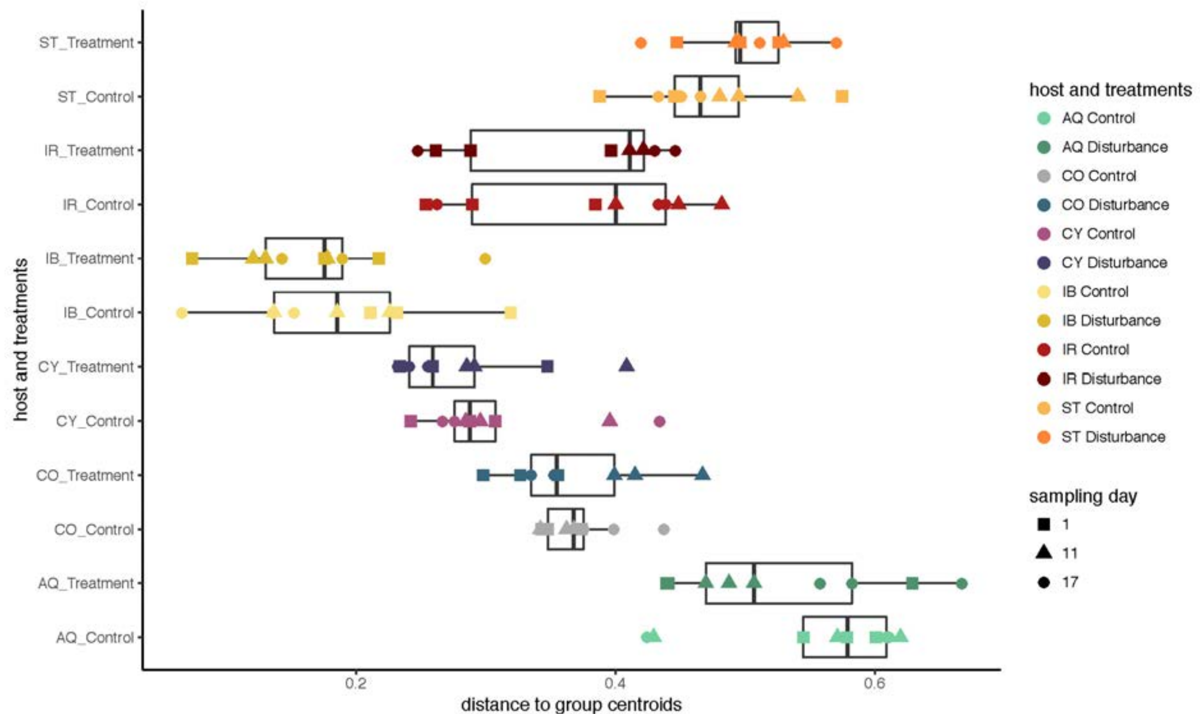

Figure 5.4. Microbiome variability (heterogeneity) for *Amphimedon queenslandica* (AQ), *Coscinoderma matthewsi* (CO), *Cymbastela coralliophila* (CY), *Ianthella basta* (IB), *Ircinia ramosa* (IR) and *Stylissa flabelliformis* (ST) under both treatment conditions (control and disturbance) including all sampling points (day 1, day 11 and day 17). Distance to group centroid (also referred to as dispersion), is used to describe heterogeneity in the microbiome.

Fine-scale variations in sponge microbiomes

Sponge microbiomes were dominated by sequences classified to the phyla *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes* and PAUC34f (Figure 5.5A). The ten most abundant ASVs for each sponge species are represented in Figure 5.5A and the ASV composition for selected taxa is shown for each host genotype in Figure 5.5B. *A. queenslandica* was dominated by seven genera belonging to the phyla *Proteobacteria* and *Bacteroidetes* (Figure 5.5A). *Nitrosococcus* (phylum *Proteobacteria*) was the most abundant genus and was represented by four ASVs (Figure 5.5B). Each *A. queenslandica* host genotype was associated with a specific *Nitrosococcus* community (ANOSIM, p = 0.001., R = 0.7128), which displayed high temporal stability irrespective of treatment. *C. matthewsi* was dominated by six genera belonging to *Proteobacteria*, PAUC34f, *Chloroflexi* and *Acidobacteria* phyla (Figure 5.5A). The three most abundant PAUC34f ASVs retrieved from the *C. matthewsi* microbiome were equally abundant in all host genotypes, except genotype CO_D which was dominated by a single PAUC34f ASV (Figure 5.5C). *C. coralliophila* was dominated by seven genera belonging to the phyla *Proteobacteria*, *Cyanobacteria* and *Chloroflexi* with the

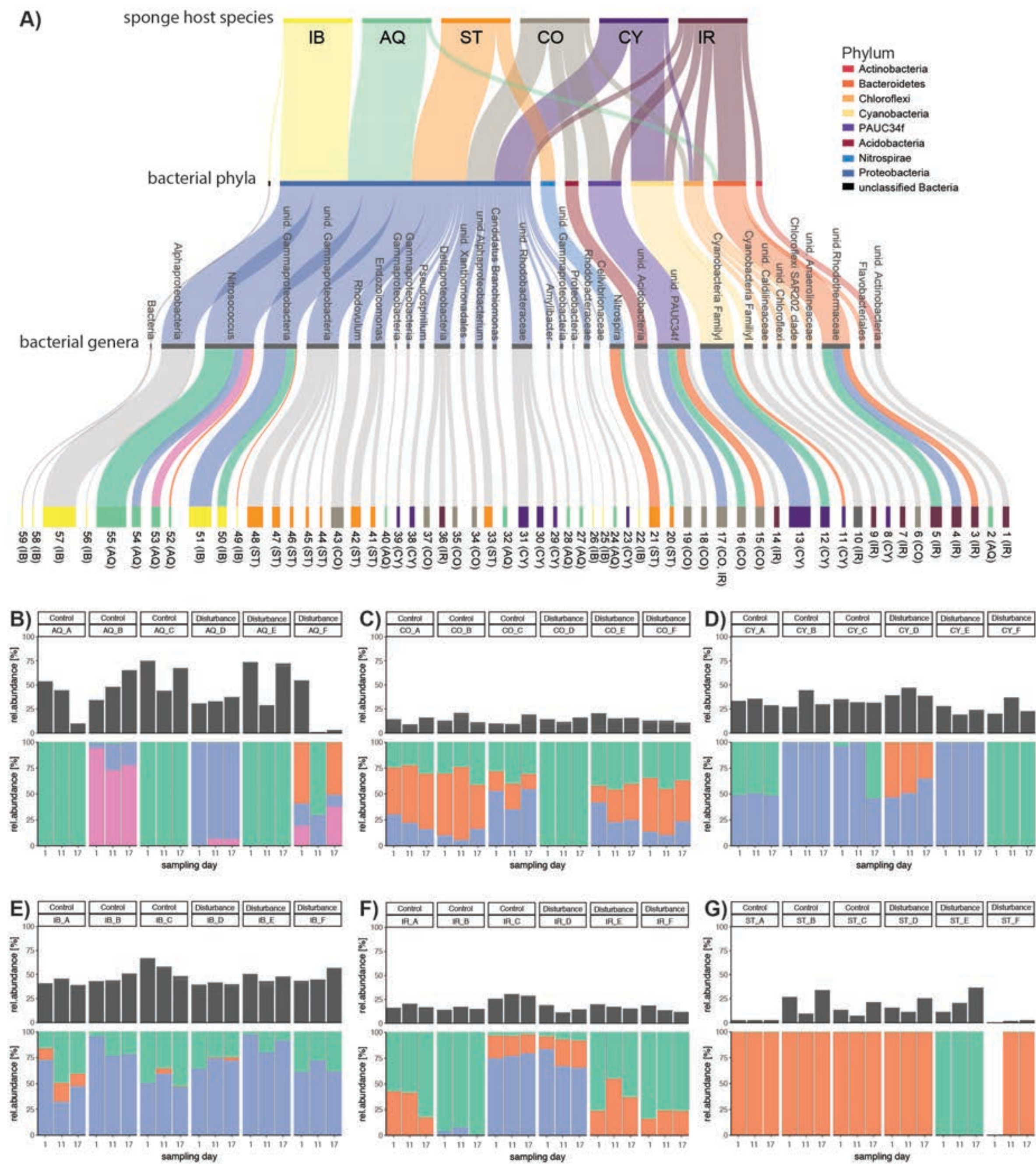

Figure 5.5. A) Alluvial diagram depicting taxonomic affiliation of the ten most abundant Amplicon Sequence Variants (ASV) associated with each sponge species (AQ = *Amphimedon queenslandica*, CO = *Coscinoderma matthewsi*, CY = *Cymbastela coralliophila*, IB= *Ianthella basta*, IR= *Ircinia ramosa* and ST= *Stylissa flabelliformis*). Colour of ASV nodes represent host species (AQ= green, CO= grey, CY= purple, IB= yellow, IR= red, ST= orange). B-G) Fine-scale compositional variation of selected bacterial taxa associated with host genotypes. B) *Nitrosococcus* ASV associated with AQ genotypes. C) PAUF34f ASV associated with CO genotypes. D) *Cyanobacteria* Family I ASV associated with CY genotypes. E) unidentified *Gammaproteobacteria* associated with IB genotypes. F) *Rhodothermaceae* ASV associated with CY genotypes and G) *Nitrospira* ASV associated with ST genotypes.

cyanobacterial ASVs revealing high host genotype specificity and high temporal stability irrespective of treatment (Figure 5.5A and 5.5D). *I. basta* was dominated by one *Alphaproteobacteria*-affiliated sequence across all genotypes while the other dominant class, *Gammaproteobacteria*, consisted of two equally abundant ASVs and a third low abundant ASV

which was not present across all host genotypes (Figure 5.5A and 5.5E). *I. ramosa* was dominated by seven bacterial genera belonging to six phyla, with the most abundant members belonging to *Rhodothermaceae* (phylum *Bacteroidetes*) (Figure 5.5A). *Rhodothermaceae* ASVs varied significantly between the *I. ramosa* host genotypes but were stable within each genotype (Figure 5.5F). The *S. flabelliformis* microbiome was dominated by the phyla *Proteobacteria* and *Nitrospirae* (Figure 5.5A), with the two dominant *Nitrospira* ASVs displaying similar relative abundance patterns across all genotypes except ST_E (Figure 5.5G).

Discussion

Disturbance of the global climate system as a result of increased green-house gas emissions is predicted to result in stronger storm activity and larger flooding events (Arnell and Gosling 2016). For near-shore coral reefs, large floods can result in acute salinity fluctuations that impact the health of marine invertebrates such as corals and sponges (Jones and Berkelmans 2014, VanWoesik et al 1995). For example, a flood plume associated with tropical cyclone "Tash" in 2011 caused a dramatic salinity drop (reaching extremes of 6.5 psu) on coral reefs in Keppel Bay (GBR, Australia) which resulted in large-scale coral mortality (Jones and Berkelmans 2014). Similar salinity extremes and mortalities were observed after cyclone "Joy" crossed the Queensland (Australia) coast in 1991, where salinity during the flood peak reached 7 - 10 psu at the surface and 15 - 28 psu at 3 m depth (VanWoesik et al 1995). However, despite experiencing an average annual salinity of ~35.7 psu in the field (Wolanski 1994), sponge species assessed in this study were highly tolerant of short-term acute salinity fluctuations (minimum of 25 psu), showing no visual signs of health deterioration, no changes in the concentration or composition of photopigments and no shifts in the sponge-associated microbial communities. The only previous assessment of salinity tolerance in sponges showed that *Cymbastela concentrica* tolerated long-term exposure to salinities ranging from 30.6 psu to 34.5 psu (Roberts et al 2006). These results contribute to an increasing body of evidence showing high environmental tolerance in sponges (Bell et al 2013, Bennett et al 2017).

The diversity-stability hypothesis posits that high diversity systems are more stable than low diversity systems upon environmental fluctuation (McCann 2000). Applying this diversity-stability paradigm to sponge microbiomes subjected to acute salinity disturbance revealed no shift in the compositional stability (e.g., compositional resistance, resilience and sensitivity differences) of the microbiome for both high (HMA) and low (LMA) diversity species. Temporal stability in HMA- and LMA-sponge microbiomes has been described along natural seasonal fluctuations (Erwin et al 2015) and sponge microbiomes have also been shown to be

resistant to sub-lethal increases in nitrogen, temperature, sediment, light and pollution (Gantt et al 2017, Luter et al 2014, Pineda et al 2016, Pineda et al 2017, Simister et al 2012, Thoms et al 2003). Furthermore, sponge microbiomes can remain stable during stress-induced tissue regression of the host (Luter et al 2012b). However, once a compositional and functional shift of the sponge-associated microbiome occurs, host mortality can rapidly follow (Blanquer et al 2016, Fan et al 2013, Webster et al 2008), highlighting the crucial link between microbial stability and host health. In addition to altering the abundance and/or prevalence of microorganisms, environmental disturbances can also induce changes to the community dispersion/heterogeneity (Zaneveld et al 2017). The recently coined Anna Karenina principle postulates that disturbances often lead to more stochastic community structures (Zaneveld et al 2017), which can be measured by the increase in multivariate dispersion of a microbiome. In our study the dispersion of microbial communities also remained consistent across both high and low diversity species, irrespective of experimental treatment. Stability in the composition and dispersion of sponge-microbial associations under short-term salinity stress emphasizes the high fidelity of sponge-microbial partnerships. Furthermore, equal compositional resistance across high and low microbial diversity species during environmental fluctuations shows that the stability of sponge microbiomes remains unaffected by its diversity. While the diversity-stability concept does not appear to hold for sponge microbiomes, it remains to be seen whether the environmental tolerance of other reef species such as corals is linked to microbiome diversity. Furthermore, the effect of microbial diversity on functional stability of sponge microbiomes remains to be determined.

Oligotyping sequence clustering techniques identify nucleotide variations (up to one nucleotide) between sequences and hence increase the ability to detect fine-scale variations, which can be informative about ecological niches, temporal dynamics and population structures (Eren et al 2014, Eren et al 2015, Mackey et al 2017). In this study, oligotyping revealed that host genotype significantly controls fine-scale bacterial composition (ASV level), whereas sponge species structures the associated bacterial genera, and the HMA-LMA dichotomy appears to influence the microbiome composition at the phylum level (Figure 5.5). For example, low microbial diversity species (*A. queenslandica*, *I. basta* and *S. flabelliformis*) were predominantly associated with bacteria belonging to the phylum Proteobacteria. In contrast, high microbial diversity species (*C. matthewsi*, *C. coralliophila* and *I. ramosa*) were associated with a complex community dominated by *Proteobacteria*, PAUC34f, *Chloroflexi*, *Bacteroidetes*, *Actinobacteria* and *Acidobacteria*. Similar observations have been reported for other LMA and HMA sponge species (Moitinho-Silva et al 2017) and results are also consistent with previous reports of high species-specificity in sponge microbiomes (Erwin et al 2015, Schmitt et al 2012, Taylor et al 2004, Thomas et al 2016). Here we further report that sponge

microbiomes also exhibit strong genotype-specificity, detected using fine-scale compositional variation at the ASV level. This is consistent with other host-microbe systems including the human gut (Blekhman et al 2015, Dabrowska and Witkiewicz 2016, Spor et al 2011) and the *Drosophila* microbiome (Chaston et al 2016, Early et al 2017). Considering the significant microbiome differences amongst host genotypes, we argue that future research on sponge microbiomes should take genotype-specific microbiome variations into account. The significant influence of host genotype on the fine-scale composition of a sponge microbiome further suggests that host intrinsic factors (e.g. host genetics) rather than environmental factors are particularly important in shaping the sponge microbiome.

Marine ecosystems, such as coral reefs, are increasingly impacted by local and global stressors (Hughes et al 2017a) and effective monitoring and management are critical to their protection. Microbial diagnostics have recently been proposed as a rapid and sensitive way to monitor environmental fluctuations in coral reef ecosystems (Glasl et al 2017). As ecologically important filter feeders with well-established microbial partnerships (Bell 2008, Bell et al 2013, Lesser 2006), sponges represent a relevant target for microbial based monitoring approaches. However, the high stability of sponge microbiomes towards a variety of natural fluctuations (Erwin et al 2012, Erwin et al 2015) and stressors (Gantt et al 2017, Pineda et al 2017, Simister et al 2012), in conjunction with fine-scale compositional variation between host genotypes, suggests that sponge-associated microbes are not suitable indicators for assessing perturbations to reef ecosystem health. Here we have also shown that the primary driver of the remarkable stability in sponge-associated microbial communities is environmental resistance rather than resilience.

Chapter 6

DISENTANGLING THE EFFECT OF HOST-GENOTYPE AND ENVIRONMENT ON THE MICROBIOME OF THE CORAL *ACROPORA TENUIS*

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Abstract

Genotype-specific contributions to the environmental tolerance and disease susceptibility of corals are widely accepted. Yet our understanding of how host genotype influences the composition and stability of the coral microbiome subjected to environmental fluctuations is limited. To gain insight into the community dynamics and environmental stability of microbiomes associated with distinct coral genotypes, we assessed the microbial community associated with *Acropora tenuis* under single and cumulative pressure experiments. Experimental treatments comprised either a single pulse of reduced salinity (minimum of 28 psu) or exposure to the cumulative pressures of reduced salinity (minimum of 28 psu), elevated seawater temperature (+ 2°C), elevated pCO_2 (900 ppm) and the presence of macroalgae. Analysis of 16S rRNA gene amplicon sequence data revealed that *A. tenuis* microbiomes were highly host-genotype specific and maintained high compositional stability irrespective of experimental treatment. On average, 48 % of the *A. tenuis* microbiome was dominated by *Endozoicomonas*. Amplicon sequence variants (ASVs) belonging to this genus were significantly different between host individuals. Although no signs of stress were evident in the coral holobiont and the vast majority of ASVs remained stable across treatments, a microbial indicator approach identified 26 ASVs belonging to *Vibrionaceae*, *Rhodobacteraceae*, *Hahellaceae*, *Planctomycetes*, *Phylobacteriaceae*, *Flavobacteriaceae* and *Cryomorphaceae* that were significantly enriched in corals exposed to single and cumulative stressors. While several recent studies have highlighted the efficacy of microbial indicators as sensitive markers for environmental disturbance, the high host-genotype specificity of coral microbiomes may limit their utility and we therefore recommend meticulous control of host-genotype effects in coral microbiome research.

Introduction

Corals contain abundant and diverse communities of microorganisms that together form a holobiont (Rohwer et al 2002). The photoautotrophic dinoflagellate endosymbionts of the family Symbiodiniaceae are by far the best studied symbiotic partners of reef-building corals. Symbiodiniaceae lineages vary between coral species (Smith et al 2017) and even between host genotypes of conspecific corals (Brener-Raffalli et al 2018). Fine-scale adaptations of the Symbiodiniaceae lineages can influence the environmental sensitivity of their hosts (Baker 2003), as some Symbiodiniaceae lineages are more thermo-tolerant and hence infer higher bleaching tolerance to corals (Rowan 2004). Corals also harbour diverse communities of bacteria, archaea and viruses (Bourne et al 2016, Hernandez-Agreda et al 2017, Thurber et al 2017). Excessive environmental stress resulting in coral bleaching, tissue necrosis and

mortality, is often accompanied by a shift in the microbiome (Glasl et al 2016, Zaneveld et al 2017). While the importance of the microbiome to coral fitness is well appreciated (Bourne et al 2016, Grottoli et al 2018, Peixoto et al 2017, Ziegler et al 2017), the microbiome's potential to expand the environmental tolerance of coral holobionts via microbial shuffling and switching is far less understood (Webster and Reusch 2017). *Endozoicomonas,* a bacterial genus commonly associated with marine invertebrates, is considered a putative symbiont of corals as it can occur at high abundance in aggregates within the tissue (Neave et al 2016) and loss of *Endozoicomonas* is frequently seen in bleached or diseased corals (Bayer et al 2013, Glasl et al 2016). Pangenome analysis of *Endozoicomonas* has revealed evidence for functional specificity between strains (Neave et al 2017b), hence fine-scale changes in the composition or relative abundance of different *Endozoicomonas* strains may contribute to variation in the environmental tolerance and disease susceptibility of conspecific corals.

A fundamental question in microbiome research is whether host intrinsic factors (e.g. genetics) or the environment are the main drivers of microbiome composition and stability (Spor et al 2011, Wullaert et al 2018). The influence of host genetics and environmental factors on the community composition of a microbiome varies between host species and even between host compartments. For example, the rizhosphere microbiome of the perennial plant *Boechera stricta* are predominantly shaped by environmental factors, however, its leaf associated microbial community is largely controlled by host genetic factors (Wagner et al 2016). Host-genotype specific factors also shape the gut microbiome of *Drosophila melanogaster*, a model system for animal-microbe interactions, and further mediate its nutritional phenotype (Chaston et al 2016). While many coral microbiome studies have focused on the effect of environmental stress (e.g., elevated temperature, increased macroalgae abundance, anthropogenic pollution and declining water quality (Garren et al 2009, Vega Thurber et al 2009, Zaneveld et al 2016, Zhang et al 2015); the combined influence of host-genotype and environment on the microbial community composition remains largely unknown. This is a critical knowledge gap as microbiome-by-host genotype-by-environment interactions may have important implications for the resistance of corals to stress and disease. Considering the recent declines in coral reefs (De'ath et al 2012, Hoegh-Guldberg et al 2007, Hughes et al 2017a) and the key role microorganisms play in maintaining host health (Bourne et al 2016), disentangling the effect of environment and host-genotype on a coral's microbiota is of utmost importance.

This study investigated the effect of host genotype-by-environment interactions on the microbiome of *Acropora tenuis*. The compositional variability of the *A. tenuis* microbiome associated with distinct host genotypes (individual coral colonies) was assessed with high taxonomic resolution based on amplicon sequence variants (ASV). The stability of the

microbiome was further investigated by exposing corals to acute salinity fluctuations (ranging from 35 psu to 28 psu) under current (sea surface temperature of 27.5°C and pCO_2 of 400 ppm) and future (sea surface temperature of 29.5°C, pCO_2 of 900 ppm and macroalgae) projected reef conditions. Stress treatments were designed to simulate environmental conditions that *A. tenuis* can experience in their natural environment. Both stress treatments (single and cumulative stress) consisted of a non-lethal low salinity pulse, mimicking freshwater influx into the reef as occurs after large rainfall events, often linked to cyclones that cross the Eastern Australian coastline and result in large riverine flows into the nearshore and mid-shelf reef areas of the Great Barrier Reef (e.g., Jones and Berkelmans 2014, VanWoesik et al 1995).

Materials and Methods

Coral colony collection and experimental design

Nine *Acropora tenuis* colonies were collected from Davies Reef (Great Barrier Reef, Australia) in March 2017 and transported to the National SeaSimulator at the Australian Institute of Marine Science (Townsville, Australia). Corals were fragmented into coral nubbins, glued onto aragonite plugs and kept at control temperature (27.5°C) and light (150 mol photons m^{-2} s^{-1}) conditions in indoor flow-through aquaria for three weeks to allow healing. Corals were collected under the permit G12/35236.1 granted by the Great Barrier Reef Marine Park Authority to the Australian Institute of Marine Science.

The experimental design consisted of three treatment conditions: 1) control, 2) single stress and 3) cumulative stress treatment (Figure 6.1). Nubbins of all nine *A. tenuis* genotypes (A-I) were exposed to all three treatment conditions to explore microbiome variation according to host genotype. Each experimental aquarium (three aquaria per treatment) held nubbins of three *A. tenuis* genotypes (four nubbins per genotype, total of 12 nubbins per aquarium). Coral nubbins were acclimated to experimental aquaria for three weeks during which corals in the cumulative stress treatment were gradually ramped to 29.5°C and 900 ppm pCO_2 over a period of 12 days. Corals in the control and single stressor treatments were kept at stable temperature (27.5°C) and ambient (400 ppm) pCO_2 conditions throughout the experiment.

Salinity was ramped down over 3 h to a minimum of 28 psu and oscillated between 28 psu and 30 psu in a six-hour rhythm to simulate natural fluctuations occurring on reefs (tidal influences). Temperature and $pCO2$ adjusted freshwater (0.2 µm filtered) was used to lower salinities prior to supplying the low saline seawater to the aquaria tanks. After seven days of low salinity, the salinity was ramped up (3 h) to 35 psu. In the cumulative stress treatment, corals were additionally exposed to elevated temperature (29.5°C), pCO_2 (900 ppm) and

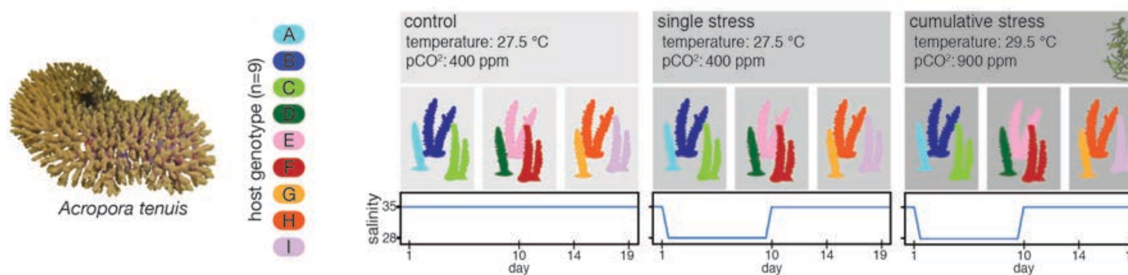

Figure 6.1. Conceptual overview of the experimental design. *Acropora tenuis* colonies (n = 9) were fragmented and coral nubbins of each host genotype (A-I) were exposed to three different treatment conditions (control, single stress and cumulative stress) and sampled on a regular basis throughout the experiment (day 1, day 10, day 14 and day 19).

macroalgae (*Sargassum* sp.), as predicted for the end of the 21st century (IPCC 2014).

Samples were collected regularly throughout the experiment (see Figure 6.1), including 24 h before the salinity pulse was induced (day 1) and at three time points (day 10, day 14 and day 19) after the low-salinity stress exposure. All nubbins were processed as follows: effective quantum yield was measured (pulse amplitude modulation fluorometry), photographed, inspected for visual signs of stress (tissue lesions, bleaching and necrosis), rinsed with 0.2 µm filter-sterilized seawater, snap frozen in liquid nitrogen and stored at -80°C until further processing.

Coral nubbins were defrosted on ice before tissue was removed with an airgun in 10 mL 1 x PBS (pH = 7.4), homogenised for 1 min at 12,500 rpm with a hand-held tissue homogeniser (Heidolph Silent Crusher M) and subsequently aliquoted for the quantification of Symbiodiniaceae cell density, chlorophyll *a*, protein concentration and DNA extraction for amplicon-based sequencing of the 16S rRNA gene. Aliquots (500 µL) for Symbiodiniaceae cell counts were fixed with formaldehyde (final concentration 1.5 %) and stored in the dark at room temperature. Aliquots for chlorophyll *a*, protein and DNA extraction (1 mL each) were centrifuged for 10 min at 16,000 g, the supernatant was discarded and the remaining pellet was snap frozen in liquid nitrogen and stored at -80°C until further processing. Coral nubbin surface area was assessed by a single paraffin wax dipping for 2 s followed by 5 min air-drying. The weight of each coral nubbin before and after dipping was quantified and the surface area was calculated against a standard curve.

Physiology of Symbiodiniaceae and the coral holobiont

The effective quantum yield of the Symbiodiniaceae was measured using PAM fluorometry. Corals were light adapted (5 h) before measuring the response of the photosystem II effective quantum yield (ΔF/Fm') with a Heinz Walz™ Imaging PAM as previously described (Chakravarti et al 2017). Coral nubbins were exposed to a saturation pulse and the minimum and maximum fluorescence was recorded and effective quantum yield was calculated.

Symbiodiniaceae cell densities were manually counted under a stereomicroscope using formaldehyde fixed tissue samples (final concentration 1.5 %). Samples were briefly vortexed and 9 µL of each sample was added to either side of two haemocytometers and the density of symbiont cells was quantitatively normalised to the tissue blastate and aliquot volume, and standardised to the nubbin's surface area.

Chlorophyll *a* was extracted and concentrations were measured using a spectrophotometric assay. Tissue pellets were defrosted on ice, centrifuged at 16,000 g for 10 min at 4 °C, and remaining supernatant was discarded. Pellets were re-suspended in 1 mL of 100 % acetone and incubated in the dark for 24 h at 4 °C after which they were centrifuged at 16,000 g for 10 min and supernatant (200 µL) was pipetted into a 96-well plate in triplicate. Absorbance at 630 nm and 663 nm was measured using a BioTek microplate reader and chlorophyll *a* concentration was calculated (see Appendix C Equation S6.1), quantitatively normalised to the tissue blastate and aliquot volume, and standardised to the nubbin's surface area.

Total protein concentration was quantified using a Pierce™ BCA Protein Assay kit (Thermo Scientific) following the manufacturer's instruction. Absorbance was measured in triplicate for each sample at 562 nm in a BioTek Plate reader. Standard curves were calculated using a bovine serum albumin (BSA) solution, creating a working range between 20 and 2000 µg mL^{-1} and total protein was calculated against the BSA standard curve, quantitatively normalised to the tissue blastate and aliquot volume, and standardised to the surface area of each individual nubbin.

DNA extraction, 16S rRNA gene sequencing and analysis

DNA of all coral samples was extracted using the DNeasy PowerBiofilm kit (QIAGEN) following the manufacturer's instructions. Blank extractions were included to control for kit contamination. Coral DNA extracts were stored at -80°C until shipment on dry ice to Ramaciotti Centre (University of New South Wales, Australia) for sequencing. The V1-V3 region of the 16S rRNA gene was amplified using primers 27F (5`- AGAGTTTGATCMTGGCTCAG -3`; Lane 1991) and 519R (5`-GWATTACCGCGGCKGCTG -3`; Turner et al. 1999) and libraries were prepared with the Illumina TruSeq protocol, followed by Illumina MiSeq 2 x 300 bp sequencing (see Appendix C Table S6.1).

Demultiplexed paired end reads were analysed in QIIME2 (Version 2017.9.0; https://qiime2.org) as previously described by Glasl et al (2018b). In brief, forward and reverse reads were truncated at their 3' end at the 296 and 252 sequencing positions, respectively. Samples were checked for chimeras and grouped into features based on 100 % sequence similarity, from here on referred to as ASV (amplicon sequence variants), using DADA2

(Callahan et al 2016). Multiple *de novo* sequence alignments of the representative sequences were performed using MAFFT (Katoh et al 2002). Non-conserved and highly gapped columns from the alignment were removed using default settings of the mask option in QIIME2. Unrooted and rooted trees were generated for phylogenetic diversity analysis using FastTree. For taxonomic assignment, a Naïve-Bayes classifier was trained on the SILVA v123 99 % Operational Taxonomic Units, where reference sequences only included the V1-V2 regions (27F/519R primer pair) of the 16S rRNA genes. The trained classifier was applied to the representative sequences to assign taxonomy. A total of 11,063,364 reads were retrieved from 100 sequenced samples and clustered into 4,624 ASVs (Table 6.1). Chloroplast and mitochondria derived sequence reads and singletons were removed from the dataset and the feature table was rarefied to an even sequencing depth of 3,506 sequencing reads, leading to the exclusion of four samples. Demultiplexed sequences and metadata are available from the NCBI Sequence Read Archives (SRA) under accession number PRJNA492377.

Table 6.1. Sequencing and sample overview.

Host-genotype	Total no. of samples	No. of sequences	Richness[a]	Evenness[a]	Shannon Index[a]
A	12	54,352 (±18,259)	71 (± 64)	0.63 (± 0.05)	2.53 (± 0.48)
B	12	31,702 (±19,058)	51 (± 44)	0.66 (± 0.14)	2.49 (± 0.86)
C	12	26,421 (± 26,065)	108 (± 86)	0.73 (± 0.11)	3.23 (± 0.65)
D	12	59,543 (± 28,560)	101 (± 102)	0.64 (± 0.07)	2.74 (± 0.80)
E	12	27,348 (± 24,386)	100 (± 110)	0.69 (± 0.10)	2.97 (± 0.81)
F	12	36,097 (± 21,293)	108 (± 103)	0.73 (± 0.08)	3.18 (± 0.84)
G	4	55,460 (± 35,822)	126 (± 74)	0.75 (± 0.07)	3.46 (± 0.74)
H	12	44,101 (± 19,488)	92 (± 63)	0.65 (± 0.14)	2.81 (± 0.64)
I	12	51,998 (± 23,968)	109 (± 73)	0.63 (± 0.08)	2.82 (± 0.65)

[a)] diversity indices (average ± SD) for each host genotype are based on a non-rarefied ASV table from which chloroplast and mitochondria derived reads were removed

Statistical analysis

Statistical analysis was performed in R (R Development Core Team 2008). Holobiont health metadata were z-score standardized and variation between treatments and host genotypes was evaluated using Analysis of Variance (ANOVA) and if applicable, variations were further assessed with a Tukey post-hoc test. Multivariate statistical approaches including Multivariate Homogeneity of Group Dispersion ('vegan package'; Oksanen et al 2013), Permutation Multivariate Analysis of Variance (PERMANOVA, 'vegan package'; Oksanen et al 2013), Non-metric multidimensional scaling (NMDS 'phyloseq package'; McMurdie and Holmes 2013) and distance based Redundancy Analysis (dbRDA 'phyloseq package'; McMurdie and Holmes 2013) were based on Bray Curtis dissimilarities. Mantel statistics based on Pearson's product-moment correlation (mantel test, 'vegan package'; Oksanen et al 2013) were used to evaluate whether sample-to-sample dissimilarities in microbiome composition and physiological

holobiont health parameters (protein concentration, chlorophyll *a* concentration, Symbiodiniaceae cell densities and effective quantum yield) were correlated. Holobiont health parameters were z-score standardised and dissimilarity matrices were based on Bray Curtis dissimilarities.

Alpha diversity measures including richness and Shannon diversity for the *Endozoicomonas* community were analysed using the 'phyloseq package' (McMurdie and Holmes 2013). Variation in the total relative abundance of all *Endozoicomonas* ASVs per sample between treatments, over time and between host-genotypes was assessed using ANOVAs with arcsine-square-root transformed relative abundance data. The phylogenetic tree of the 11 most abundant *Endozoicomonas* ASVs was produced with phyloseq (McMurdie and Holmes 2013) using the Newick rooted tree generated in QIIME2 (Version 2017.9.0; https://qiime2.org).

Indicator value analysis (IndVal, 'indispecies" package; De Cáceres and Legendre 2009) was used to identify ASVs significantly associated with treatment groups (control, single stress and cumulative stress) based on their occurrence and abundance distribution. Day 1 samples were excluded from the IndVal analysis to restrict the dataset to ASVs significantly associated with coral tissue after stress exposure (day 10, day 14 and day 19).

Graphs were created in R using ggplot2 (Wickham 2009) and phyloseq packages (McMurdie and Holmes 2013). Alluvial diagram was generated in RAWGraph (Mauri et al 2017).

Results

Coral holobiont physiological response

Corals showed no visual signs of stress (change in pigmentation, bleaching, tissue necrosis and/or mortality) in any treatment. Chlorophyll *a* concentrations remained stable between treatments (one-way ANOVA with sampling time point as blocking factor, $F_{(2/94)} = 2.707$, $p = 0.072$), however, effective quantum yield (ΔF/Fm'; one-way ANOVA with sampling time point as blocking factor, $F_{(2/94)} = 15.52$, $p = 1.49 \times 10^{-6}$), symbiont cell densities (one-way ANOVA with sampling time point as blocking factor, $F_{(2/94)} = 8.83$, $p = 3.06 \times 10^{-4}$) and protein concentration (one-way ANOVA with sampling time point as blocking factor, $F_{(2/94)} = 5.563$, $p = 5.21 \times 10^{-3}$) varied significantly between treatments within sampling time points (Figure 6.2A). Coral nubbins in the cumulative stress treatment contained significantly lower protein and symbiont cell densities, while displaying significantly higher effective quantum yield compared to nubbins in the control and single stressor treatments (Tukey Posthoc test, Appendix C Table S6.2). Furthermore, effective quantum yield (one-way ANOVA, $F_{(8/91)} = 2.688$, $p = 0.0106$),

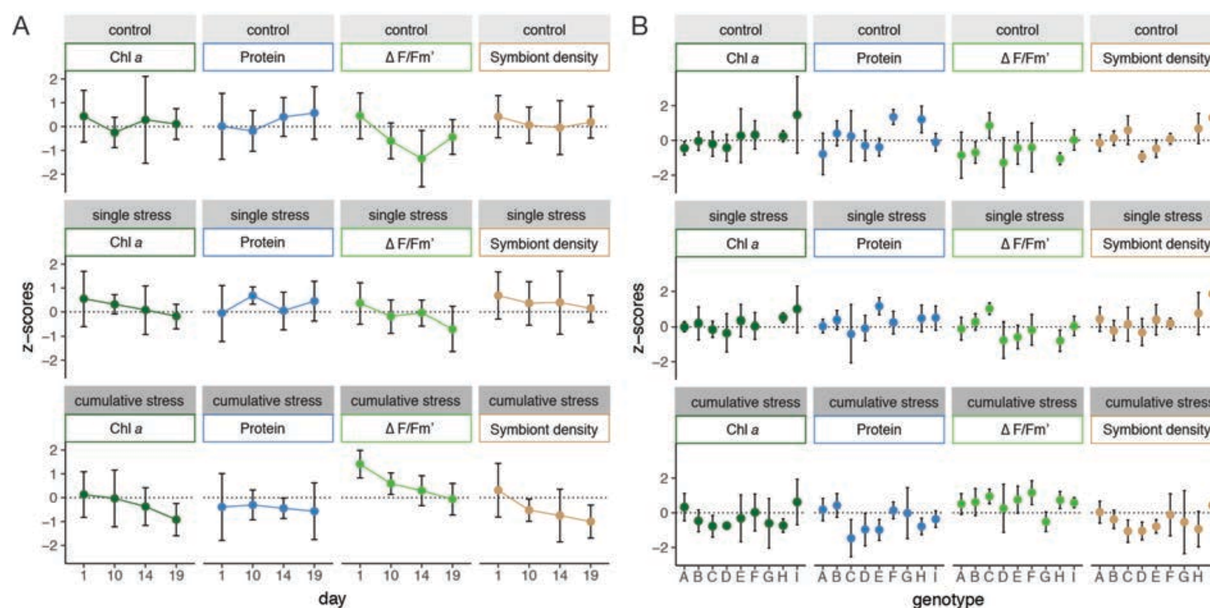

Figure 6.2. Physiological response of *Acropora tenuis* under control, single stress and cumulative stress treatments. Variations in the chlorophyll *a* (Chl *a*) concentration, protein concentration, effective quantum yield (Δ F/Fm') and symbiont cell density (Symbiont density) of *A. tenuis* (A) over time (day 1, 10, 14 and 19) and (B) between individual host-genotypes (A-I). Physiological parameters are z-score standardised and error bars represent standard deviations.

symbiont cell densities (one-way ANOVA, $F_{(8/91)} = 4.334$, $p = 1.86 \times 10^{-4}$) and chlorophyll *a* concentrations (one-way ANOVA, $F_{(8/91)} = 2.773$, $p = 8.64 \times 10^{-3}$) varied significantly between host genotypes (Figure 6.2B). Protein concentration, however, was unaffected by host genotype (one-way ANOVA, $F_{(8/91)} = 1.783$, $p = 0.0906$) and hence was the only holobiont health parameter solely affected by treatment.

Microbial community response

The microbiome of *A. tenuis* remained highly stable across treatments, with no significant changes in the heterogeneity, also referred to as multivariate dispersion (one-way ANOVA, $F_{(2/93)}= 1.2107$, $p = 0.3026$; Figure 6.3A), or in community composition (PERMANOVA, $p = 0.5156$, 10,000 permutations; Figure 6.3B). However, the microbiome composition varied significantly between individual host genotypes (PERMANOVA, $p = 9.99 \times 10^{-5}$, 10,000 permutations), but was unaffected by treatment, sampling time point or tank effects when tested for each genotype individually (PERMANOVA with host-genotype as blocking factor, 10,000 permutations, Appendix C Table S6.3). Similar results were obtained using presence/absence data (Appendix C Figure S6.1). Host genotype was the only significant factor, explaining 32.4 % of the observed community variation (permutational ANOVA for dbRDA based on 1,000 permutations, $p = 9.99 \times 10^{-4}$; Appendix C Figure S6.2). Treatment and holobiont health parameters did not significantly contribute to the microbiome variation (Appendix C Table S6.4). Furthermore, no significant correlation between similarity matrices based on microbiome composition and physiological holobiont health parameters

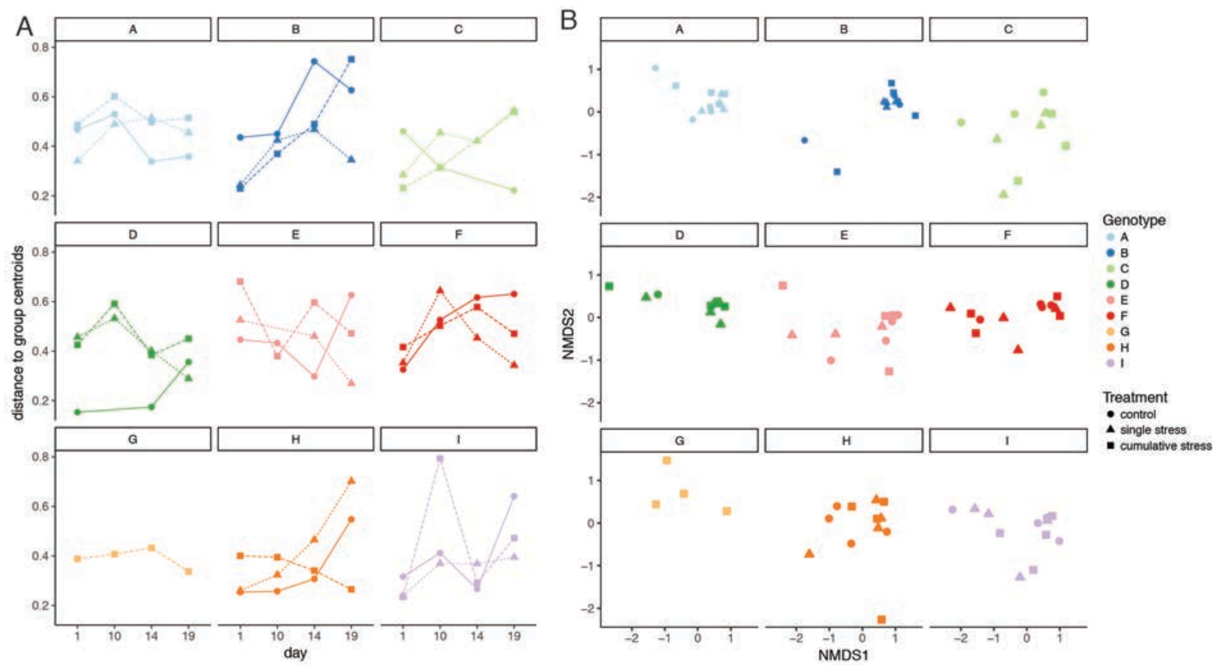

Figure 6.3. Configurational and compositional stability of *Acropora tenuis* microbiome. (A) Multivariate dispersion (heterogeneity) measured by the distance to the group centroid for each host-genotype (A-I) within each treatment (control, acute stress and cumulative stress) over time (day 1, 10, 14 and 19). (B) Non-metric multidimensional scaling (NMDS) illustrating compositional similarity of sample replicates of each host-genotype (A-I) under different treatment conditions (control, single stress and cumulative stress).

was observed (chlorophyll *a*, protein, effective quantum yield and symbiont cell density; Mantel statistic based on Pearson's product-moment correlation $r = -0.0238$, $p = 0.6243$, 10,000 permutations).

Endozoicomonas assemblage

Endozoicomonas affiliated sequences comprised the majority of the *A. tenuis* microbiome, representing 48 % (± 29 %) of the community (based on proportion of reads) and comprising 133 unique ASVs. One *Endozoicomonas* strain (ASV 11) was consistently present (100 % of all samples) and highly abundant (19 % ± 12 %) throughout the experiment (Figure 6.4). The *A. tenuis* microbiome also contained diverse bacteria affiliated with phyla including *Proteobacteria* (30 %), *Actinobacteria* (10 %), *Firmicutes* (2.4 %) and *Bacteroidetes* (1.9 %; Figure 6.4).

The total relative abundance of *Endozoicomonas* was not affected by treatment (two-way ANOVA, $F_{(2/84)} = 0.473$, $p = 0.625$), sampling time point (two-way ANOVA, $F_{(3/84)} = 0.588$, $p = 0.625$) or the interaction of treatment-by-sampling time point (two-way ANOVA, $F_{(6/84)} = 0.696$, $p = 0.654$). However, total relative *Endozoicomonas* abundance varied significantly between host genotypes (one-way ANOVA, $F_{(8/87)} = 3.741$, $p = 2.04 \times 10^{-4}$) and remained stable between treatments when tested for each genotype individually (within subject ANOVA, $F_{(2/85)} = 0.756$, $p = 0.473$); Figure 6.5A).

The *Endozoicomonas* community composition also varied significantly between host genotypes (PERMANOVA, p = 9.99 x 10^{-5}, 10,000 permutations, Figure 6.5), however, was unaffected by treatment, sampling time point or tank (PERMANOVA with host-genotype as blocking factor, 10,000 permutations, Appendix C Table S6.5). Furthermore, host-genotype significantly explained 26.4 % of the observed compositional variability of the *Endozoicomonas* community (permutational ANOVA for dbRDA based on 1,000 permutations, p = 9.99 x 10^{-5}; Figure 6.5B).

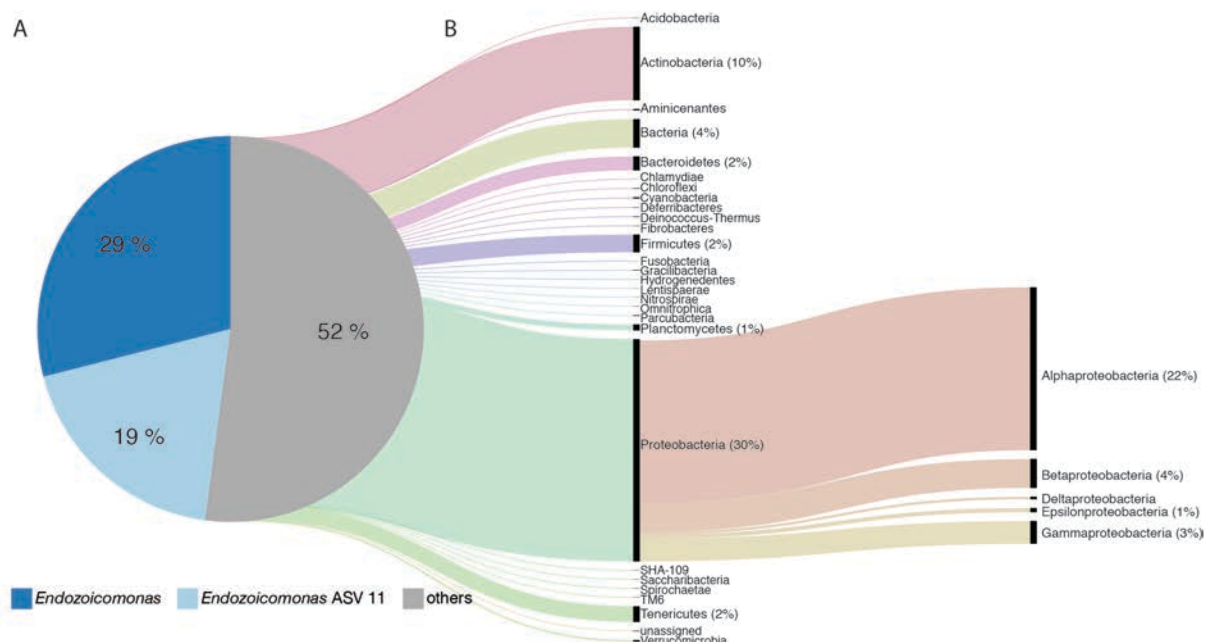

Figure 6.4. The taxonomic composition of the *Acropora tenuis* microbiome. (A) The *A. tenuis* microbiome was dominated by the bacterial genus *Endozoicomonas* (average relative abundance of 48%), with one *Endozoicomonas* ASV (ASV 11) present in all samples (average relative abundance of 19%). (B) The average contribution of the remaining microbiome (others) is displayed as an alluvial diagram, depicting the proportional contribution of bacterial phyla (classes for *Proteobacteria*). Mean relative abundances (%) are provided for bacterial taxa >1 %.

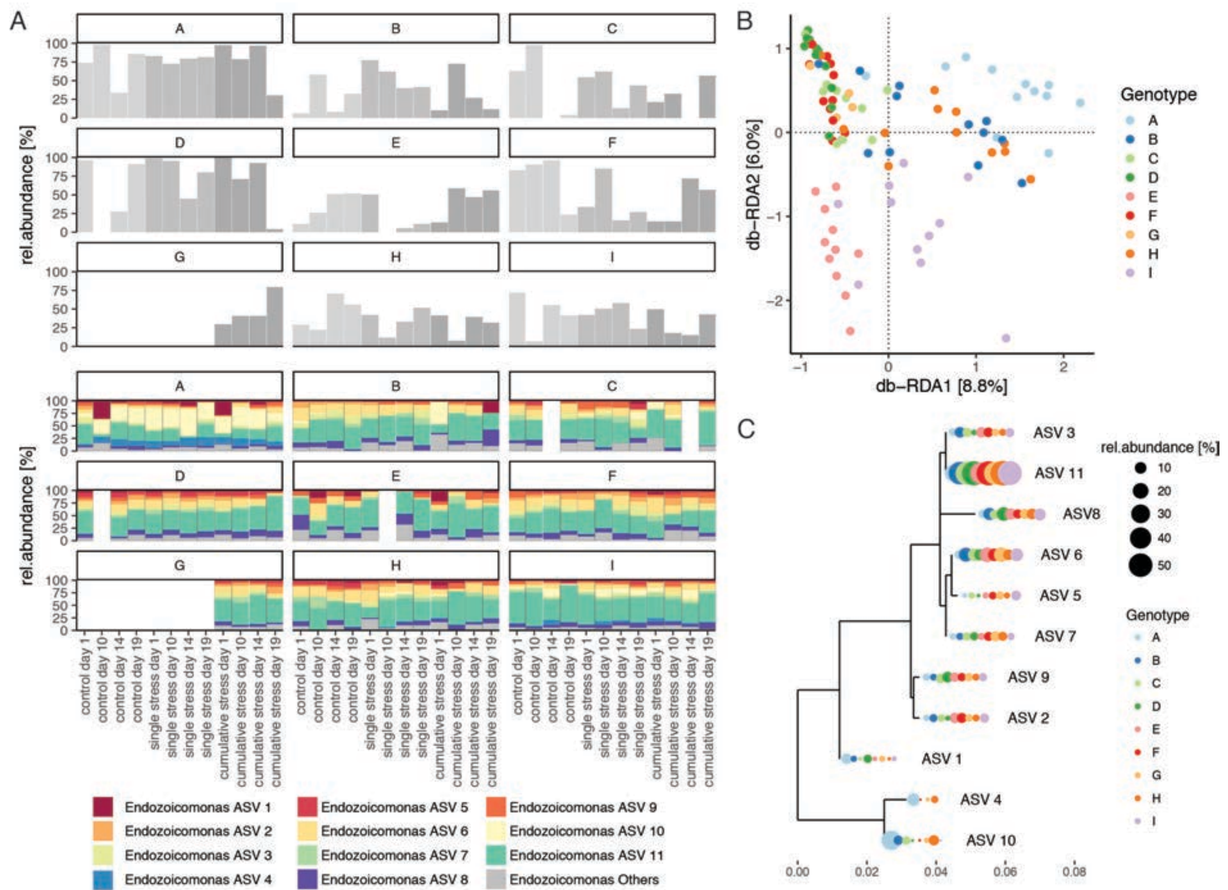

Figure 6.5. Composition and distribution of *Endozoicomonas* assemblages. (A) Total relative abundance of *Endozoicomonas* and the relative abundance distribution of the 11 most abundant *Endozoicomonas* amplicon sequence variants (ASVs) associated with individual coral nubbins of each host genotype (A-I) under control, single stress and cumulative stress conditions over time (day 1, 10, 14 and 19). (B) Distance-based Redundancy Analysis (dbRDA) quantifying the contribution of host-genotype to significantly explaining the observed compositional variation of the *Endozoicomonas* microbiome. (C) Phylogenetic tree of the 11 most abundant *Endozoicomonas* ASVs (including the ubiquitously present ASV 11) and their average relative abundance within a host genotype.

Microbial indicators for environmental stress

Indicator value analysis was performed to assess if specific ASVs could be identified as indicators for environmental stress treatments. Despite the vast majority of ASVs (i.e. 4,598 ASVs) showing no response to experimental treatment, 26 ASVs were significantly associated ($p < 0.05$) with one and / or two treatment groups (Figure 6.6, Appendix C Table S6.6). The identified indicator ASVs belonged to the bacterial families *Vibrionaceae*, *Rhodobacteraceae*, *Hahellaceae* (genus *Endozoicomonas*), *Planctomycetes*, *Phylobacteriaceae*, *Flavobacteriaceae* and *Cryomorphaceae* (Figure 6.6).

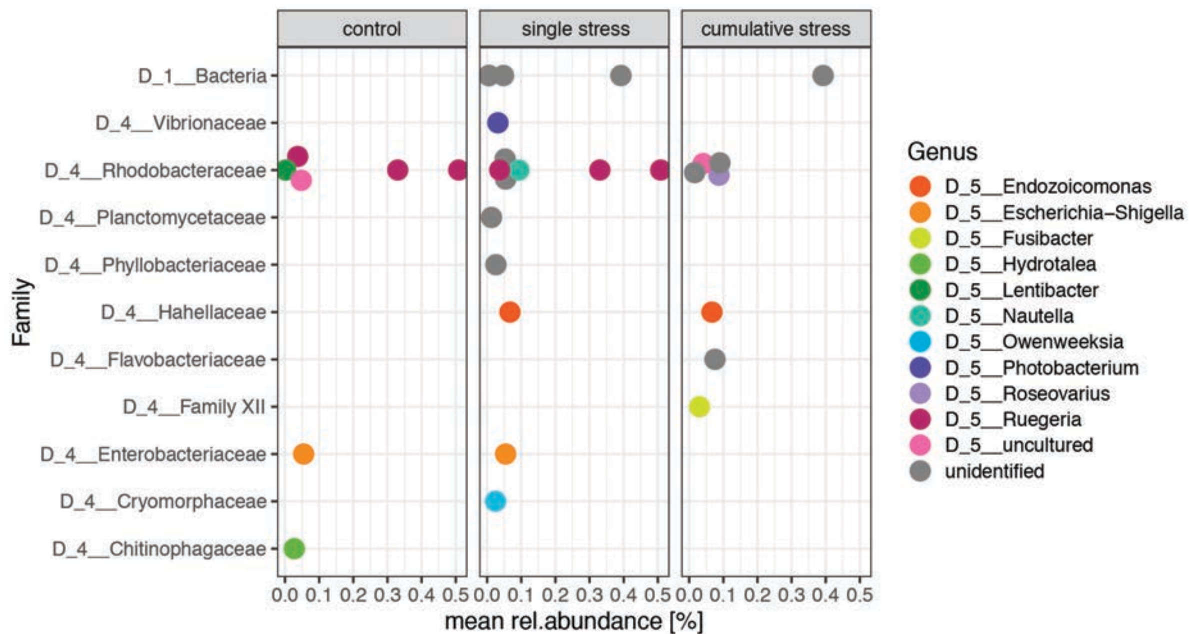

Figure 6.6. Microbial indicators significantly associated with one and / or two treatments. Indicators were identified based on their occurrence and abundance in coral tissue post stress exposure (excluding samples collected at day 1) using Indicator Value analysis. Each dot represents a single amplicon sequence variant (ASV), labelled with the taxonomic affiliation and their average relative abundance in the treatment group.

Discussion

Elucidating the effect of host genotype on microbiome composition and understanding consequences of environmental change for holobiont stability is central to predicting the influence of host genetics on the stress tolerance of corals. Here we followed the compositional stability of microbiomes associated with nine distinct *A. tenuis* genotypes when exposed to control, single and cumulative stress treatments over time. The *A. tenuis* microbiome varied significantly between coral genotypes, with host genotype being a much stronger driver of microbiome variation than environment. Similar host-genotype specificities have recently been described for sponge microbiomes (Glasl et al 2018b) and are also frequently reported for plant, crustacean and human microbiomes (Balint et al 2013, Macke et al 2017, Spor et al 2011). Traditional coral health parameters targeting the coral algal symbiont (i.e., chlorophyll *a* concentrations, symbiont cell densities, effective quantum yield) were also significantly affected by host-genotype, although no correlation between these parameters and the microbiome was observed. This suggests that the *A. tenuis* microbiome composition remains largely unaffected by the performance and density of the algal symbiont, and that other host intrinsic factors (e.g. genetics) and/or the environmental life-history of individual genotypes fine-tune the microbiome composition.

Endozoicomonas form symbiotic partnerships with diverse marine invertebrates (Neave et al 2016). In corals, *Endozoicomonas* occur as dense clusters within the coral tissue

and in some bacterial 16S rRNA gene profiling studies they can reach relative abundances as high as 95% of retrieved sequences (Bayer et al 2013, Neave et al 2016, Pogoreutz et al 2018). Loss of *Endozoicomonas* from the coral microbiome has been correlated with negative health outcomes for the coral host, though their direct effects on host fitness are unknown (Bourne et al 2008, Glasl et al 2016, Ziegler et al 2016). In *A. tenuis*, we detected no significant change in the relative frequency, alpha diversity, richness and community composition of *Endozoicomonas* following exposure to non-lethal environmental stress. These results are consistent with findings for *Pocillopora verrucosa*, where *Endozoicomonas* remains the dominant symbiont even under bleaching conditions (Pogoreutz et al 2018). In our study, the *Endozoicomonas* community generally exhibited high host-genotype specificity at the ASV level, though a single *Endozoicomonas* strain (ASV 11) was consistently shared among all coral nubbins and genotypes (including field control samples – data not shown). This ubiquitous strain likely represents a stable and consistent member of the resident *Endozoicomonas* community. A stable core is often described as a key feature of a symbiotic coral microbiome (Ainsworth et al 2015, Hernandez-Agreda et al 2017), and despite being ubiquitously persistent between conspecific corals, the core characteristically only comprises a few members of the whole microbiome (Hernandez-Agreda et al 2018).

While the *Endozoicomonas* community as a whole was not significantly affected by environmental treatment, one *Endozoicomonas* ASV was identified as a significant indicator for environmental stress. Similar environmental sensitivity has been reported for two prevalent *Endozoicomonas* species following exposure to elevated dissolved organic carbon (Pogoreutz et al 2018). Although these *Endozoicomonas* affiliated ASVs show high sequence identity, small variations in the rRNA gene sequence can impact the biology and pathogenicity of bacteria (Cilia et al 1996, Fukushima et al 2002), hence single nucleotide variations (ASV level) may affect the functional role of microbes with flow on consequences for the coral holobiont. Shuffling and switching of *Endozoicomonas* strains may therefore provide the coral holobiont with an enhanced capacity to cope with shifting environmental conditions (Neave et al 2017b), although characterisation of the symbiotic contribution made by *Endozoicomonas* to the coral host is required to better understand the ecological significance of these findings.

Recent studies have highlighted the potential for coral microbiomes to act as sensitive markers for environmental disturbance (Glasl et al 2017, Roitman et al 2018). Here we showed that a small number of ASVs, including taxa commonly reported to increase under host stress (i.e., *Vibrionaceae*, *Rhodobacteraceae*; Ben-Haim et al 2003, Bourne et al 2016, Sunagawa et al 2010), were significantly associated with the tissue of *A. tenuis* exposed to single and cumulative stress treatments. Furthermore, several unknown bacteria were also identified to be significantly associated with the tissue of *A. tenuis* exposed to single and cumulative stress

treatments. Hence, future research may focus on the identification of yet undescribed bacterial taxa associated with corals. However, despite the potential diagnostic value of these ASVs, host genotype overwhelmed any overarching effect of environment on the coral microbiome. This high divergence in the microbiome between conspecific corals is likely to hinder our ability to detect fine-scale variation of sensitive microbial indicator taxa. Therefore, unless host-genotype independent microbial indicators can be identified and validated, the efficacy of integrating microbial community data into coral health monitoring initiatives appears unfeasible due to high compositional variability between microbiomes of conspecific corals.

Conclusion

This study shows that the *A. tenuis* microbiome varies significantly between host individuals (genotypes) and that these genotype-specific communities persist during exposure to non-lethal environmental disturbances. Consideration of microbiome-by-host genotype-by-environment effects is therefore needed to elucidate how intraspecific variations of the microbiome affect the susceptibility of corals to environmental stress and disease. Furthermore, microbial variability between individual coral genotypes may cloud our ability to identify universal microbial changes during periods of adverse environmental conditions. This is particularly relevant if establishing sensitive microbial indicators for sub-lethal environmental disturbances (tested in this study), since the observed stability of the coral microbiome combined with the host genotype specificity likely precludes the robust assignment of microbial indicators across broad scales.

Chapter 7

Comparative Genome-Centric Analysis Reveals Seasonal Variation in the Function of Coral Reef Microbiomes

This chapter is submitted as

Glasl B, S Robbins, PR Frade, E Marangon, PW Laffy, DG Bourne and NS Webster (under review in the ISME Journal) Comparative genome-centric analysis reveals seasonal variation in the function of coral reef microbiomes.

Abstract

Microbially mediated processes contribute to coral reef resilience yet, despite extensive characterisation of microbial community variation following environmental perturbation, the functional stability of reef microbiomes is poorly understood. We undertook metagenomic sequencing of sponge, macroalgae and seawater microbiomes from an inshore coral reef (Magnetic Island, Great Barrier Reef) to define their functional potential and evaluate shifts in microbially mediated processes upon environmental perturbation. In total, 125 high quality metagenome-assembled genomes were reconstructed, spanning 15 bacterial and 3 archaeal phyla. Multivariate analysis of the relative abundance of genomes revealed changes in the functional potential of coral reef microbiomes in relation to seasonally mediated macroalgae biomass and temperature fluctuations. For example, a shift from *Alphaproteobacteria* to *Bacteroidota*-dominated seawater microbiomes with increasing macroalgae biomass and seawater temperature resulted in an increased genomic potential to degrade algal-derived polysaccharides. Increasing seawater temperature also resulted in an 85% reduction of *Chloroflexota* in the sponge microbiome, with potential consequences for nutrition, waste product removal and detoxification in the sponge holobiont. A shift in the *Firmicutes*:*Bacteroidota* ratio on macroalgae over summer revealed a biofilm succession that could affect polysaccharide degradation in macroalgal microbiomes. These results highlight how environmental factors can alter the functional potential of coral reef microbiomes.

Introduction

Coral reef ecosystems are being challenged by anthropogenic pressures resulting in unprecedented rates of decline (De'ath et al 2012, Hoegh-Guldberg 2014, Hughes et al 2017a). The cumulative effects of climate change (e.g., ocean warming and ocean acidification) and local pressures (e.g., overfishing and eutrophication) reduce the resilience of coral reef ecosystems (Hughes et al 2003, Hughes et al 2018a) and lead to a transition from healthy, coral-dominated ecosystems to degraded reefs, often characterised by enhanced macroalgae biomass (Bruno et al 2009, Hughes et al 2007). The increase of macroalgae in coral reef ecosystems at the expense of coral species abundance and diversity fosters a perpetuating cycle of reef degradation, hence, high macroalgae biomass is generally considered a sign of poor reef health (Haas et al 2016, Hughes et al 2007).

Microorganisms play pivotal roles in coral reefs, and the maintenance of biogeochemical cycling and microbially mediated ecological processes is considered critical for the persistence of reefs under future projected climate conditions (Ainsworth and Gates

2016, Bourne et al 2016, Webster and Reusch 2017). Cumulative environmental stressors (e.g., increased sea-surface temperatures, ocean acidification, and eutrophication) can trigger alterations in the composition and function of microbial assemblages associated with corals and sponges (Bourne et al 2008, McDevitt-Irwin et al 2017, Morrow et al 2015, Pita et al 2018, Ziegler et al 2017). Changes in the microbiomes of dominant reef-benthos can negatively impact holobiont health, with concomitant consequences for the wider reef ecosystem (Fan et al 2013, Glasl et al 2016, Ziegler et al 2017). The transition from coral to algae dominance in reef ecosystems enhances the availability of labile dissolved organic carbon (DOC) in reef waters, shifting the trophic structure towards higher microbial biomass and energy use in degraded reefs, a process termed microbialisation (Haas et al 2016, McDole et al 2012). Algae-derived DOC fosters the growth of copiotrophic, potentially pathogenic, bacterioplankton communities that can negatively impact the health of corals (Haas et al 2011, Haas et al 2016, Morrow et al 2011, Nelson et al 2013). Close proximity of macroalgae to corals can also induce shifts in the coral-associated microbial communities and potentially act as a trigger for microbial diseases (Nugues et al 2004, Sweet et al 2013, Vega Thurber et al 2012). As corals perish, more space becomes available for algae, thereby creating a positive feedback loop called DDAM; DOC, disease, algae, microorganism (Barott and Rohwer 2012, Haas et al 2016).

Metagenomics is providing new insights into the functional roles microorganisms play on coral reefs (e.g., Dinsdale et al 2008, Haas et al 2016, Vega Thurber et al 2009). However, the enormous habitat complexity of coral reefs means that microbial communities associated with different reef niches are rarely holistically assessed within a single study (Garren and Azam 2012). Given the strong benthic-pelagic coupling that occurs in coral reef ecosystems, integrated functional assessments of free-living and host-associated microbiomes are needed to better understand the contributions of microbially mediated processes to reef ecosystem health (Garren and Azam 2012, Glasl et al 2017). Furthermore, recent computational advances enable precise metabolic reconstructions of microbial genomes from complex microbial communities (Imelfort et al 2014, Kang et al 2015, Wu et al 2016). Thus, identifying how the functional potential of reef microbiomes respond to environmental perturbation (i.e. temperature) and benthic species composition (i.e., macroalgae and coral abundance) is now possible at an ecosystem scale.

This genome-centric coral reef microbiome study assessed microbial community shifts in response to seasonally-mediated fluctuations in sea-surface temperature and macroalgal abundance and evaluated the functional implications for host-associated (sponge and macroalgae) and seawater microbiomes. Coastal inshore reef systems of the Great Barrier Reef (GBR) are characterised by high macroalgal abundance (particularly the canopy-forming

brown algae *Sargassum* spp.) and reduced coral cover (De'ath and Fabricius 2010, Wismer et al 2009). *Sargassum* biomass on inshore reefs of the GBR fluctuates seasonally and reaches a maximum during early summer and a minimum during mid-winter (Ceccarelli et al 2018, Schaffelke and Klumpp 1997). Algae-dominated shallow inshore reefs of the GBR are also exposed to larger temperature fluctuations compared to off-shore reefs (Mellin et al 2019), with sea-surface temperature at inshore reefs frequently reaching 30°C during summer (Walther et al 2013). Hence, inshore regions on the GBR provide an ideal system to study the effects of macroalgae biomass and temperature fluctuations on coral reef microbiomes.

Materials and Methods

Sample collection and preparation

Marine sponge (*Coscinoderma matthewsi*), macroalgae (*Sargassum* spp.) and seawater samples for metagenomic sequencing were collected during two sampling events (August 2016 and February 2017) at Geoffrey Bay, Magnetic Island (Great Barrier Reef, Queensland, Australia). Additional seawater samples were collected in June 2016, October 2016, December 2016 and March 2017. Seawater temperatures at the sampling location ranged between 23°C in August to 30°C in February (Appendix D Figure S7.1). Macroalgae biomass followed the seasonal growth-decay pattern reported for nearby inshore reef locations (Great Palm Island, Great Barrier Reef, Queensland, Australia), where the maximum biomass of dominant macroalgae was observed in February (~200 g ash free dry weight per m^2) and the lowest biomass (~30 g ash free dry weight per m^2) in August (Schaffelke and Klumpp 1997). Samples were collected under the permit G16/38348.1 issued by the Great Barrier Reef Marine Park Authority.

Samples (n = 3 per sample type per sampling event) for metagenome sequencing were collected and processed following the standard operating procedures of the Australian Marine Microbial Biodiversity Initiative (AMMBI) as previously described (Glasl et al 2019a). In brief, seawater was collected with collapsible sterile bags at 2 m depth and pre-filtered (50 µm) to remove larger particles and subsequently filtered (2 L) onto 0.2 µm Sterivex-filters (Millipore). The sponge *Coscinoderma matthewsi* was removed from the substrate (at 7 m depth) with sterile scalpel blades, rinsed with 0.2 µm filter-sterilised seawater and subsampled into 2 mL cryogenic vials. *Sargassum* spp. was sampled with sterile scalpels at 3 m depth, rinsed with 0.2 µm filtered-sterilised seawater and placed into 2 mL cryogenic vials. All samples were immediately snap frozen in liquid nitrogen and stored at -80°C.

Prior to DNA extraction, the macroalgal biofilm was separated from the macroalgal tissue by overnight incubation at 200 rpm in 10 mL 1 x PBS at 37℃ (Glasl et al 2019a).

Microbes within sponge tissue were separated from sponge host cells as described in detail by Botte et al (2019). Briefly, sponge tissue was rinsed twice (5 min at 200 rpm on an orbital incubation shaker) with sterile calcium- and magnesium-free seawater (CMFSW) and homogenised using a handheld tissue homogeniser (Heidolph Silent Crusher M) for 10 min at 7,000 rpm in CMFSW. Next, filter sterilised collagenase (Sigma Aldrich) was added to the homogenised sponge tissue (final concentration of 0.5 mg/mL) and the tissue slurry incubated on ice for 30 min at 150 rpm on an incubation orbital shaker. After incubation, the microbial cells from the sponge tissue slurry were enriched by a series of filtration and centrifugation steps. The final microbial pellet was recovered in 1mL Tris-HCl/NaCl and stored at -20℃ until required for DNA extraction.

DNA extractions and metagenome sequencing

DNA from seawater and macroalgal biofilms was extracted with the DNeasy PowerSoil kit (QIAGEN). DNA of sponge-associated microbial cells was extracted with the DNeasy PowerBiofilm kit (QIAGEN) following the manufacturer’s instructions. DNA extracts were stored at -80℃ until shipment on dry ice to the Australian Genome Research Facility (AGRF; Melbourne, Australia) for sequencing. Libraries were prepared with the Nextera XT Library Preparation kit (Illumina), following the manufacturer’s protocol and sequenced on a HiSeq 2500 in rapid run mode with 250 bp paired-end reads (24 samples per flow cell resulting in approximately 5 to 6 Gbp per sample). Sequencing data, metadata and protocols are freely available at the Bioplatforms Australia data portal under the Australian Microbiome project (www.data.bioplatforms.com).

Read assembly, binning and de-replication

Sequence adapters of raw reads were removed using SeqPurge v2018_04 (Sturm et al 2016) and adapter trimmed reads of samples were assembled individually with metaSpades v3.13.0 (Nurk et al 2017) using default settings. Coverage files for metagenomic binning were calculated by mapping adapter-trimmed reads to assembled scaffolds using BamM v1.7.3 (https://github.com/Ecogenomics/BamM) and metagenome-assembled-genomes (MAGs) were generated with uniteM v0.0.15 (https://github.com/dparks1134/UniteM) using the following binning tools: GroopM v0.3.4 (Imelfort et al 2014), MaxBin v2.2.4 (Wu et al 2016) and MetaBAT v2.12.1 (Kang et al 2015). The quality (completeness and contamination) of the resulting MAGs was assessed with CheckM v1.0.12 (Parks et al 2015). The total recovery of MAGs with qualities ≥ 50 (contamination score = 3) was estimated with singleM v0.12.1 (https://github.com/wwood/singlem). Total number of bins recovered from sponge, algae and seawater samples, along with bin completeness, contamination and recovery is summarised in Appendix D Table S7.1. Furthermore, to calculate relative abundances, MAGs from each

habitat (sponge, macroalgae, seawater) were first de-replicated separately at 95 % Average Nucleotide Identity (95 % ANI) using dRep v1.0.0 to avoid arbitrary placement of reads between very similar MAGs (Olm et al 2017). Secondly, adapter-trimmed reads from samples collected in August 2016 and February 2017 were mapped (75 % minimum alignment and 95% minimum identity) against the de-replicated $MAGs_{95\%ANI}$ with coverM v0.2.0 (https://github.com/wwood/CoverM).

Taxonomic assignment and functional annotation of MAGs

Taxonomy was assigned to the $MAGs_{95\%ANI}$ using GTDBtk v0.2.1 (https://github.com/Ecogenomics/GTDBTk; see Appendix D Table S7.2) and functional annotations were assigned with enrichM v0.4.7 (https://github.com/geronimp/enrichM) using the Kyoto Encyclopaedia of Genes and Genomes Orthology (KEGG; KOs). KEGG defines "modules," which are collections of KOs that together make up a metabolic pathway (e.g. glycolysis) or functional unit (e.g. flagellar assembly). The completeness of KEGG Modules in the individual $MAGs_{95\%ANI}$ was assessed using the classify function of enrichM v0.4.7 (https://github.com/geronimp/enrichM) and only KEGG modules with ≥ 70 % completeness were kept in the analysis.

Seawater $MAGs_{95\%ANI}$ belonging to the phylum *Bacteroidota* were further screened for the presence of Polysaccharide Utilization Loci (PULs). To identify the presence of PULs, *Bacteroidota* $MAGs_{95\%ANI}$ were annotated with enrichM v0.4.7 using the Carbohydrate Active enzyme (CAZy) database and the Protein Family (Pfam) database to screen for glycoside hydrolase families (GH) and susD-like genes (PF07980, PF12741, PF14322 and PF12771), respectively (Krüger et al 2019).

Statistical analysis

Statistical analysis was performed in R (R Development Core Team 2008) using the following packages: vegan (Oksanen et al 2013), VennDiagram (Chen 2014), DESeq (Anders and Huber 2010) and phyloseq (McMurdie and Holmes 2013). Graphs were created in R using ggplot2 (Wickham 2009) and illustrations were created in Adobe Illustrator.

Variations in the functional profiles of sponge, algae and seawater associated $MAGs_{95\%ANI}$ (presence / absence of KEGG Modules) were evaluated using multivariate statistical approaches including Permutation Multivariate Analysis of Variance (PERMANOVA) and Non-metric multidimensional scaling (NMDS). Dissimilarity matrices of functional presence / absence profiles were generated using the binary Bray Curtis dissimilarity index. The number of unique and shared KEGG Modules associated with carbohydrate metabolism, energy metabolism and environmental information processing among sponge, macroalgae and seawater $MAGs_{95\%ANI}$ were evaluated using Venn diagrams.

Microbial taxa showing significantly different relative abundances between August (peak of winter season) and February (peak of summer season) in sponge, macroalgae and seawater samples, respectively, were evaluated using differential abundance analysis in DESeq. The number of reads mapped to $MAGs_{95\%ANI}$ was determined at the phylum level (class for *Proteobacteria*) for each sample and normalised using variance stabilisation implemented in the DESeq package.

Differences in the functional profiles of microbial taxa that varied significantly between August and February or remained stable between sampling events were further assessed using PERMANOVAs and NMDSs based on binary Bray Curtis dissimilarities. Similarity Percentage (SIMPER with 10,000 permutations) analysis was used to further pinpoint which KEGG Modules significantly contributed to the observed dissimilarities between August *versus* February enriched taxa of macroalgae and seawater microbiomes, and between winter enriched *versus* stable taxa of the sponge microbiome. Log2 fold change of significant KEGG Modules was calculated to compare the proportional changes between groups using the gtools package v3.8.1. in R. Additionally, the phylum (class for *Proteobacteria*) contributing the most to the observed change was assessed.

Results

Microbial functional diversity of macroalgae-dominated inshore reefs

A total of 125 $MAGs_{95\%ANI}$ were recovered, belonging to 15 bacterial and 3 archaeal phyla (Figure 7.1a and Appendix D Table S7.2). Seawater samples yielded the highest number of recovered microbial genomes with 67 $MAGs_{95\%ANI}$, followed by the sponge tissue with 38 $MAGs_{95\%ANI}$ and the macroalgae biofilm with 20 $MAGs_{95\%ANI}$ (Appendix D Table S7.2). Functional profiles of the recovered microbial genomes varied significantly between habitats (PERMANOVA $F_{(2/122)} = 5.24$, $p = 9.99 \times 10^{-5}$, 10,000 permutations, Figure 7.1b). Similar patterns in taxonomic and functional diversity were observed when metagenome reads were analysed with a gene-centric approach (Appendix D Figure S7.2).

KEGG Modules involved in carbohydrate metabolism, energy metabolism and processing of environmental information represented on average more than half of all KEGG Modules annotated in sponge, macroalgae and seawater $MAGs_{95\%ANI}$ (Figure 7.1c). The relative abundance of KEGG Modules associated with these three categories was highly similar between habitats (Figure 7.1c). The functional category "biosynthesis of other secondary metabolites" was only found in macroalgae $MAGs_{95\%ANI}$ (Figure 7.1c) and more specifically, referred to the ability of *Firmicutes* $MAGs_{95\%ANI}$ to biosynthesis Bacilysin (Appendix D Figure S7.3).

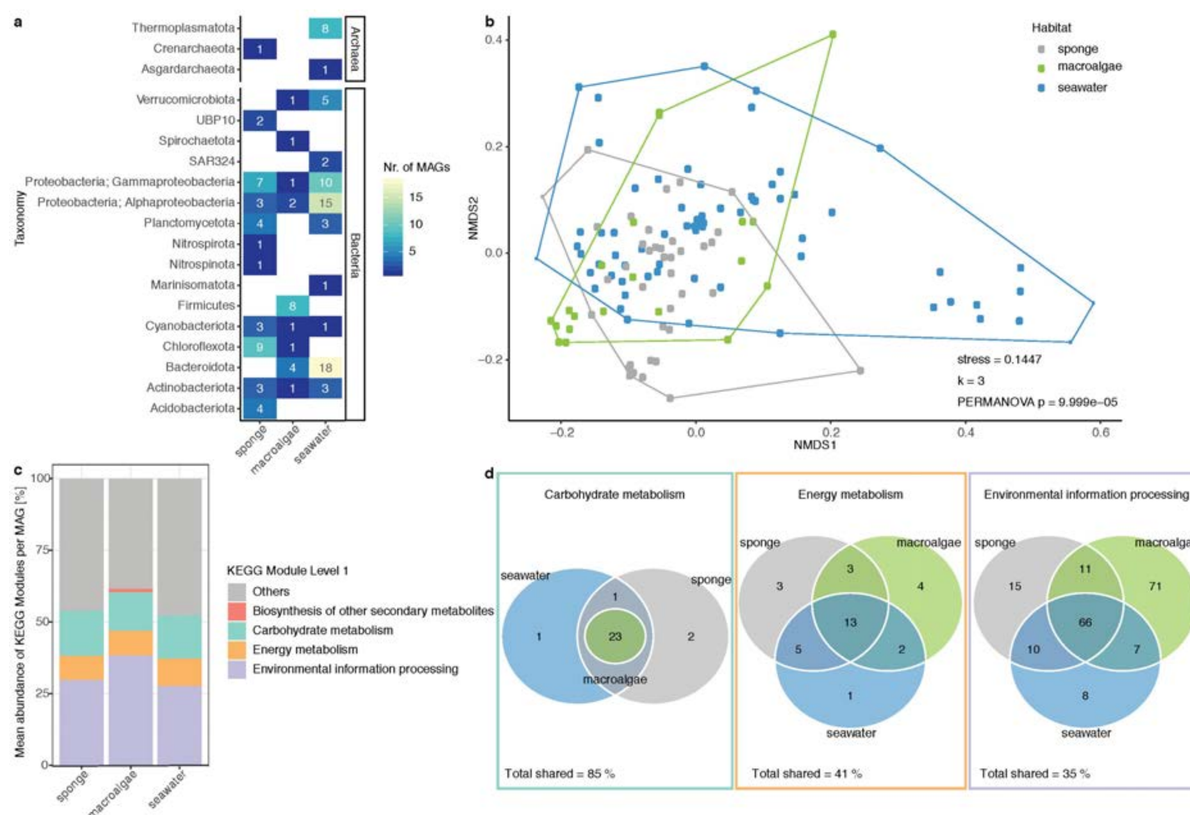

Figure 7.1. Functional diversity of metagenome assembled genomes based on 95 % average nucleotide identity ($MAGs_{95\%ANI}$) associated with sponge tissue, macroalgae biofilms and seawater. a) Total number of $MAGs_{95\%ANI}$ discovered within each bacterial and archaeal phylum (class for *Proteobacteria*). b) Non-metric multidimensional scaling plot (NMDS) based on binary Bray Curtis dissimilarities displaying the functional variability between sponge, macroalgae and seawater MAGs. c) The average composition of KEGG Modules associated with MAGs for each habitat. The three most abundant categories are shown individually as well as the unique category "Biosynthesis of other secondary metabolites" associated with macroalgae MAGs. The other eight categories are summarised as "Others". d) The absolute number of unique and shared KEGG Modules between sponge, macroalgae and seawater $MAGs_{95\%ANI}$ for carbohydrate metabolism, energy metabolism and environmental information processing (from left to right).

To further explore functional similarities between sponge, macroalgae and seawater $MAGs_{95\%ANI}$, the number of shared and unique KEGG Modules of the three main categories (carbohydrate metabolism, energy metabolism and environmental information processing processes) of each habitat was enumerated (Figure 7.1d and Appendix D Figure S7.3). In total, 85 % of annotated KEGG Modules relating to carbohydrate metabolism were shared between sponge, macroalgae and seawater $MAGs_{95\%ANI}$ (Figure 7.1d and Appendix D Figure S7.3). KEGG Modules of the central carbohydrate metabolism (i.e., glycolysis, pentose phosphate pathway, Entner-Doudoroff pathway, citrate cycle) showed 100 % overlap between habitats (Appendix D Figure S7.3). In contrast, only 41 % and 35 % of KEGG Modules related to energy metabolism and environmental information processing, respectively, were shared between sponge, macroalgae and seawater $MAGs_{95\%ANI}$ (Figure 7.1d and Appendix D Figure S7.3). Carbon fixation (such as Calvin cycle and Arnon-Buchanan cycle, also referred to as reductive citric acid cycle) and ATP synthesis KEGG Modules were highly conserved between habitats (Appendix D Figure S7.3). A higher variability between habitats was observed in their

potential to metabolise methane (i.e., formaldehyde assimilation and methane oxidation), nitrogen (i.e., assimilatory and dissimilatory nitrate reduction) and sulphur (i.e., assimilatory and dissimilatory sulphate reduction, and sulphate oxidation) as well as their potential to gain energy through photosynthesis (Appendix D Figure S7.3). The highest number of unique environmental information processing KEGG Modules was observed in the macroalgae $MAGs_{95\%ANI}$ (Figure 7.1d). These unique KEGG Modules are mainly involved in antibiotic resistance and antibiotic transport, the transfer of sugar molecules via phosphorylation (phosphotransferase system) and two-component regulatory systems for chemosensory, virulence and antibiotic biosynthesis (Appendix D Figure S7.3). Environmental information processing KEGG Modules unique to sponge $MAGs_{95\%ANI}$ (Figure 7.1d and Appendix D Figure S7.3) included copper-processing transport system, antibiotic transport and resistance, cationic antimicrobial peptide (CAMP) resistance, Type IV secretion systems and two-component regulatory systems (i.e., nitrogen fixation, nitrate respiration, metal and copper tolerance, and quorum sensing). KEGG Modules unique to seawater $MAGs_{95\%ANI}$ included transporters for Glycerol and N-Acetylglucosamine and two-component regulatory systems for glutamine utilization, C4-dicarboxylate transport, type four fimbriae synthesis, and tricarboxylic acid transport. KEGG Modules ubiquitously present in seawater, sponge and macroalgae $MAGs_{95\%ANI}$ were the ABC-2 type transport systems, aminoacyl tRNA metabolism, Twin-arginine translocation (Tat) system, Sec (secretion) system, Phosphate transport system as well as a phosphate starvation response two-component regulatory system.

Switching and shuffling of microbial taxa and functional profiles

Sponge-affiliated microbial taxa remained highly stable between winter and summer sampling events (Figure 7.2a), with only 1 of 11 taxa varying significantly (based on differential relative abundance analysis using DESeq). The phylum *Chloroflexota* (9 MAGs95%ANI) was significantly enriched in winter samples and reduced by 85% in summer (Figure 7.2b and Appendix D Table S7.3a). In conjunction, functional profiles of the phylum *Chloroflexota* differed significantly from the stable microbial community, comprised of microbial taxa that remained equally abundant between sampling time points (PERMANOVA $F_{(1/36)} = 9.56$, p = 9.99 x 10-5, 10,000 permutations; Figure 7.3). KEGG Modules driving the significant functional dissimilarity between winter and stable summer microbial taxa (based on SIMPER) were predominantly affiliated with *Chloroflexota* MAGs95%ANI (Figure 7.4). A substantial reduction of *Chloroflexota* from the sponge could have implications for the microbiomes ability to metabolise carbohydrates such as glucose and fructose, and for the ability to transfer sugar molecules between the microbiome and the host (decrease in saccharide transport systems).

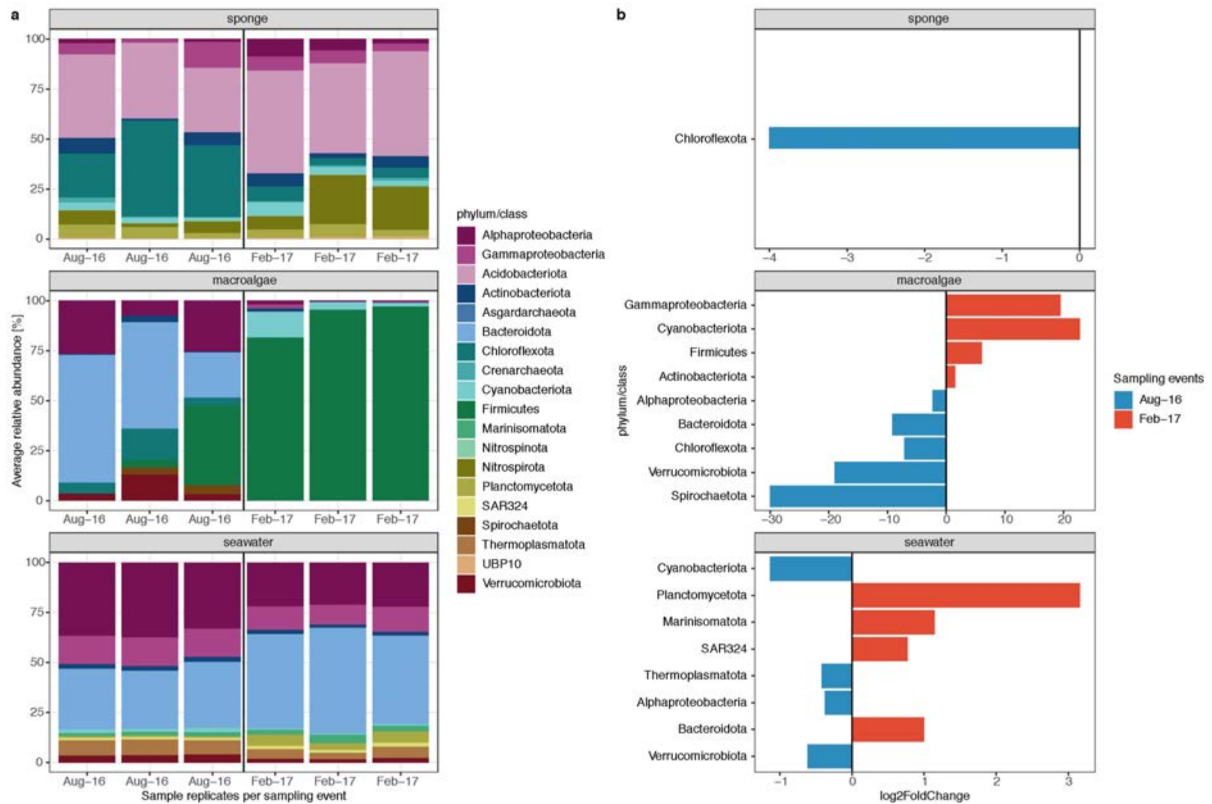

Figure 7.2. Compositional stability of microbiomes associated with sponge tissue, macroalgae biofilms and seawater between winter (August) and summer (February). a) Relative abundances of metagenome assembled genomes based on 95 % average nucleotide identity (MAGs$_{95\%ANI}$) on phylum (class for *Proteobacteria*) level in the sample replicates collected in August 2016 and February 2017. b) Log2 fold change of significantly enriched microbial phyla (class for *Proteobacteria*) between winter and summer sampling events based on differential abundance analysis (DESeq).

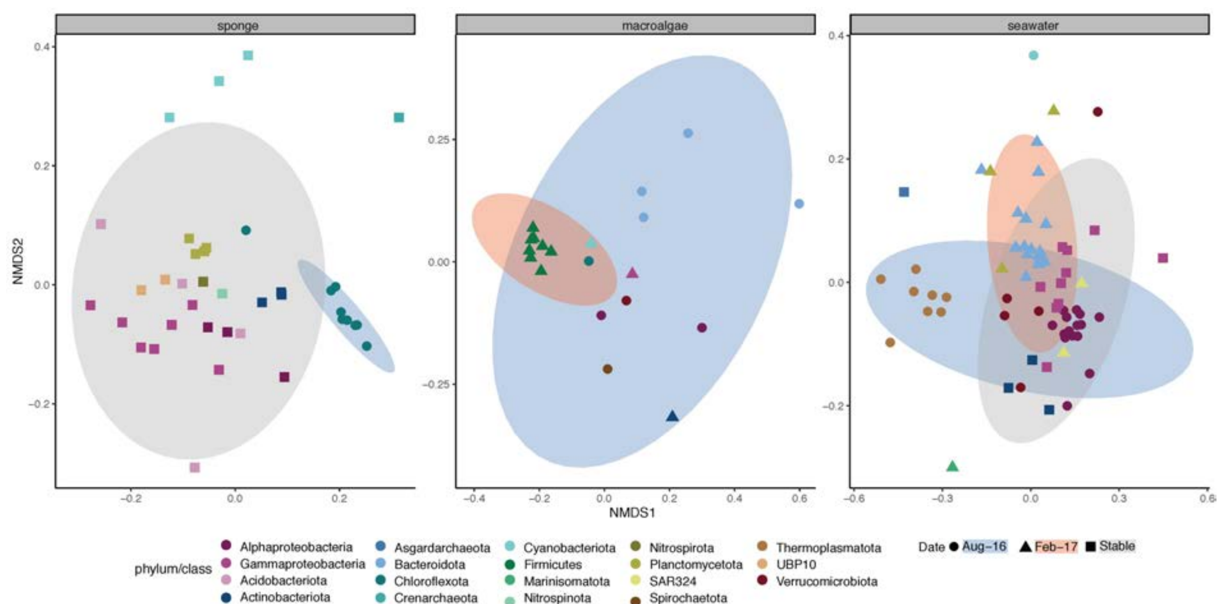

Figure 7.3. Functional profiles of metagenome assembled genomes based on 95% average nucleotide identity (MAGs$_{95\%ANI}$) associated with sponge tissue, macroalgae biofilm and seawater. Non-Metric Dimensional Scaling plot based on binary Bray Curtis dissimilarities displaying variations in the functional profiles (KEGG Module presence / absence) between MAGs$_{95\%ANI.}$ Colour represents phylum of MAGs$_{95\%ANI}$ (class for *Proteobacteria*) and shape represent whether a phylum (class for *Proteobacteria*) was significantly enriched during a sampling time point (August *versus* February) or stable between sampling time points (Stable). Hulls represent the multivariate t-distribution of groups (August, February, Stable).

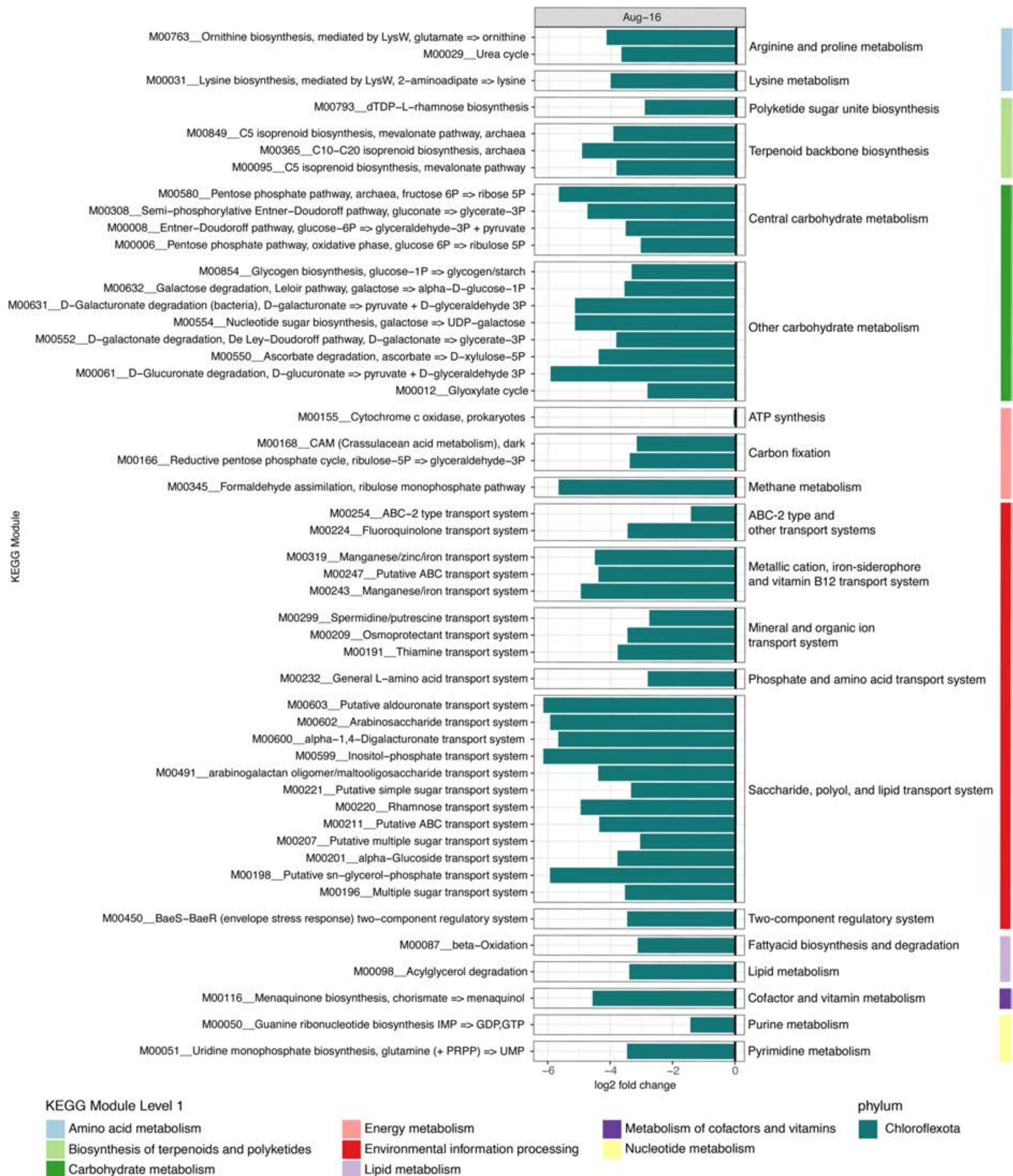

Figure 7.4. Sponge associated microbial functions significantly associated with the winter-enriched phylum *Chloroflexota*. KEGG Modules significantly ($p < 0.05$) driving the observed functional dissimilarities of enrichment groups (August *versus* Stable) were evaluated with Similarity Percentages (SIMPER). The enrichment of significant KEGG Modules is displayed as log 2-fold change. Colour indicates the microbial taxa contributing most to the observed function.

In addition, the pentose phosphate shunt, a glucose oxidation pathway, was significantly linked with *Chloroflexota* $MAGs_{95\%ANI}$. Other KEGG Modules significantly affiliated with *Chloroflexota* $MAGs_{95\%ANI}$ were Vitamin B1 (Thiamine) transport system, antibiotic transport systems (Fluoroquinolone) and metal transport systems (e.g., Manganese, Zinc and Iron).

In contrast to the sponge microbiome, macroalgae biofilm $MAGs_{95\%ANI}$ varied

significantly between sampling events (Figure 7.2a), with all 9 microbial taxa significantly enriched in either winter or summer samples (Figure 7.2b; based on differential abundance analysis using DESeq). During winter the phyla *Spirochaetota* (1 MAG$_{95\%ANI}$), *Verrucomicrobiota* (1 MAG$_{95\%ANI}$), *Bacteroidota* (4 MAGs$_{95\%ANI}$), *Chloroflexota* (1 MAG$_{95\%ANI}$) and the class *Alphaproteobacteria* (2 MAGs$_{95\%ANI}$) were significantly enriched, whereas the phyla *Actinobacteriota* (1 MAG$_{95\%ANI}$), *Firmicutes* (8 MAGs$_{95\%ANI}$), *Cyanobacteria* (1 MAG$_{95\%ANI}$) and the class *Gammaproteobacteria* (1 MAG$_{95\%ANI}$) were significantly enriched during summer (Figure 7.2b and Appendix D Table S7.3b). The significant switching and shuffling of microbial

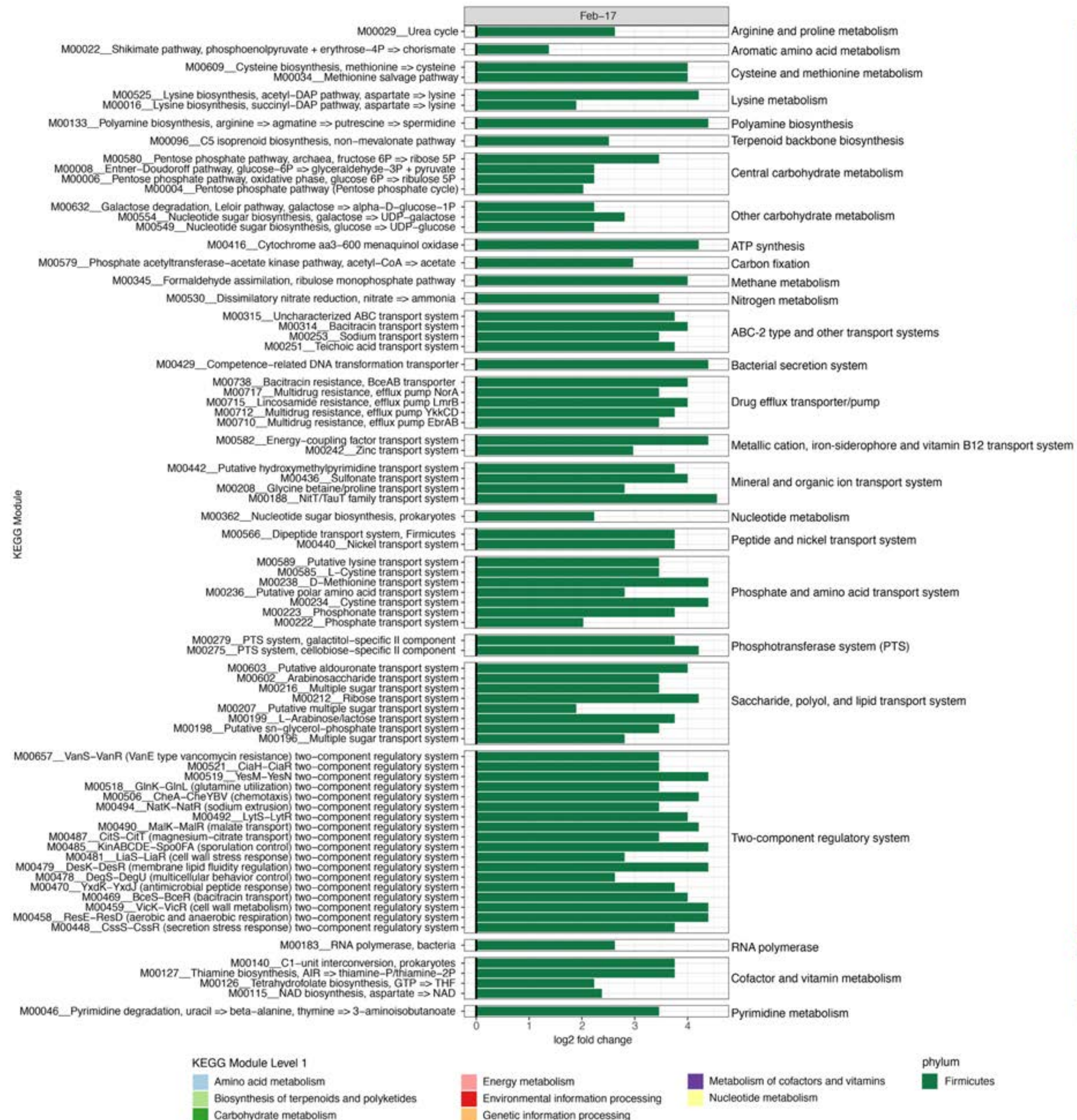

Figure 7.5. Macroalgae associated microbial functions significantly associated with summer-enriched taxa. KEGG Modules significantly ($p < 0.05$) driving the observed functional dissimilarities of enrichment groups (August *versus* February) were evaluated with Similarity Percentages (SIMPER). The enrichment of significant KEGG Modules is displayed as log 2-fold change. Colour indicates the microbial taxa contributing most to the observed function.

taxa in the macroalgae biofilm also had implications for the underlying functional repertoire (February *versus* August, PERMANOVA $F_{(1/18)} = 4.92$, $p = 9.99 \times 10^{-5}$, 10,000 permutations; Figure 7.3). *Firmicutes*, for example, were significantly enriched during summer (Figure 7.2b) and were the primary contributors to the observed functional dissimilarities between sampling time points (Figure 7.5). KEGG Modules associated with *Firmicutes* $MAGs_{95\%ANI}$ included degradation of carbohydrates (i.e., galactose and glucose) and the uptake of carbohydrates upon phosphorylation (phosphotransferase system - PTS). The observed PTSs were specific to galactitol and cellobiose. Saccharide and polyol transport systems were also enriched in *Firmicutes* $MAGs_{95\%ANI}$ (Figure 7.5). Furthermore, dissimilatory nitrate reduction (nitrate respiration) and a two-component regulatory system activating aerobic and anaerobic respiration genes were found in summer-enriched *Firmicutes* $MAGs_{95\%ANI}$. *Firmicutes*-dominated biofilms were also enriched in KEGG Modules encoding for antibiotic and multidrug resistance and transport systems as well as two component regulatory systems for antibiotic resistance, behaviour control, sporulation control and stress response (Figure 7.5).

Seawater samples, like the macroalgae biofilm, displayed high variability in abundant members between sampling events, with 8 of 11 microbial taxa varying significantly between winter and summer sampling events (Figure 7.2; based on differential abundance analysis using DESeq). The bacterial phyla *Bacteroidota* (18 $MAGs_{95\%ANI}$), SAR324 (2 $MAGs_{95\%ANI}$), *Marinisomatota* (1 $MAG_{95\%ANI}$), *Plancotmycetota* (3 $MAGs_{95\%ANI}$) were significantly enriched during summer, while the bacterial phyla *Verrucomicrobiota* (5 $MAGs_{95\%ANI}$), *Cyanobacteriota* (1 $MAGs_{95\%ANI}$), *Proteobacteria* (class *Alphaproteobacteria*; 15 $MAGs_{95\%ANI}$) and the archaeal phylum *Thermoplasmatota* (8 $MAGs_{95\%ANI}$) were significantly enriched in winter (Figure 7.2b and Appendix D Table S7.3c). Seawater microbial taxa enriched in winter, summer, and the stable microbial community (August *versus* February *versus* Stable) also exhibited significant differences in their functional profiles (PERMANOVA $F_{(2/64)} = 4.79$, $p = 9.99 \times 10^{-5}$, 10,000 permutations; Figure 7.3). These results suggest that switching and shuffling of microbial taxa between time points (Figure 7.2) leads to significant functional changes in pelagic reef microbiomes (Figure 7.3). Functional dissimilarities between sampling time points were mainly attributed to winter enriched *Alphaproteobacteria* $MAGs_{95\%ANI}$ and archaeal *Thermoplasmatota* $MAGs_{95\%ANI}$, and the summer enriched *Bacteroidota* $MAGs_{95\%ANI}$ (Figure 7.6). The increase of *Bacteroidota* $MAGs_{95\%ANI}$ led to an increase in KEGG Modules potentially linked to virulence and pathogens. For example, *Bacteroidota* $MAGs_{95\%ANI}$ were enriched in the biosynthesis of polyketide sugars (dTDP-L-rhamnose biosynthesis), KDO2-lipid A biosynthesis (Raetz pathway), lipoprotein releasing system and the Twin-arginine translocation (Tat) system. Furthermore, *Bacteroidota* $MAGs_{95\%ANI}$ showed genomic potential for Phosphatidylethanolamine (PE) biosynthesis. Aromatic amino acid metabolism (Tryptophan

metabolism) and methionine degradation (sulphur containing amino acid) were also enriched during summer. Additionally, *Bacteroidota* $MAGs_{95\%ANI}$ were all equipped with SusD-like genes and glycoside hydrolase (GH) families (Appendix D Table S7.4 and S7.5), indicating their ability to degrade polysaccharides via the PUL machinery. The GH 16 family was the most abundant GH in the *Bacteroidota* $MAGs_{95\%ANI}$.

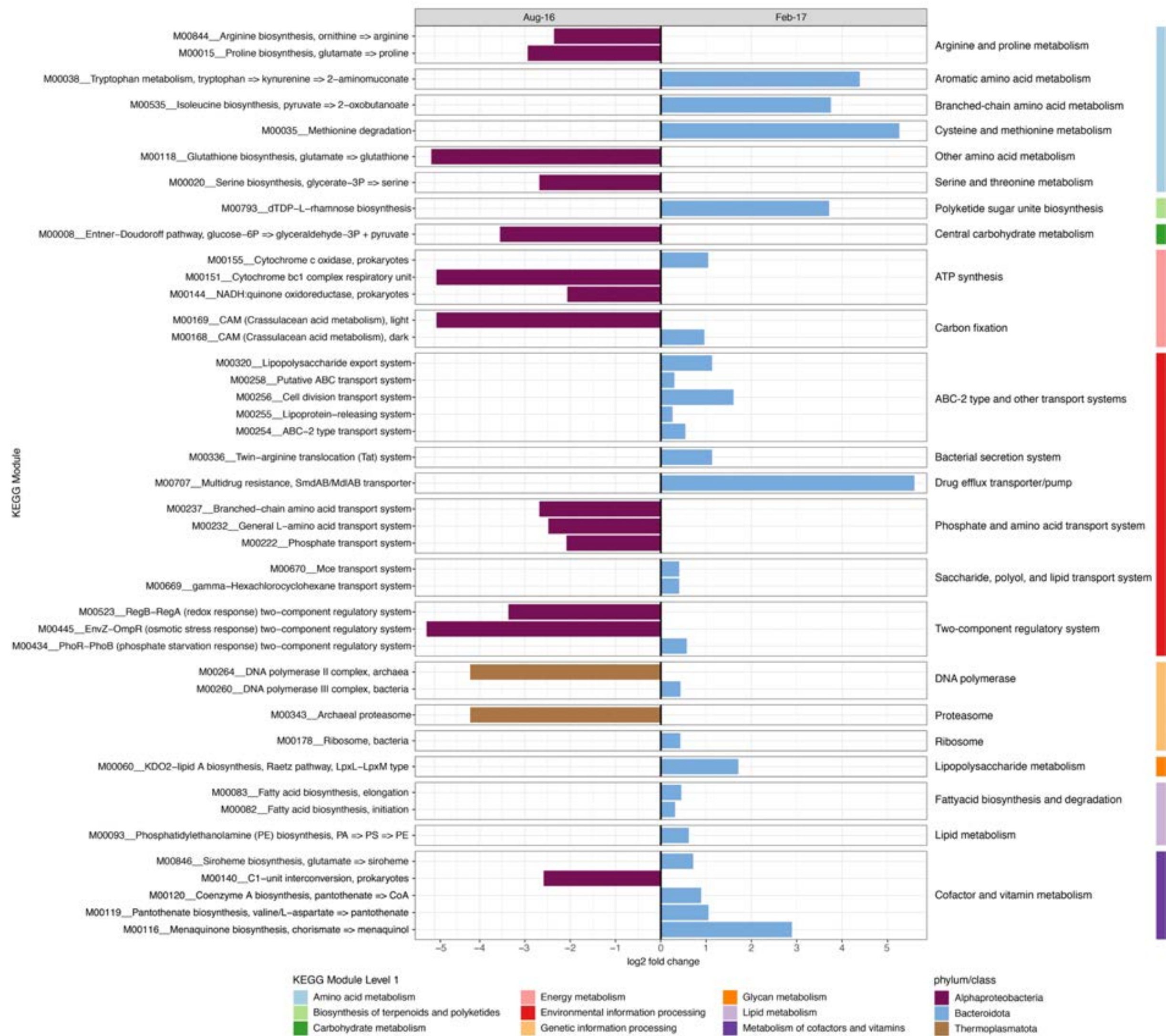

Figure 7.6. Seawater microbial functions significantly associated with winter and summer-enriched taxa. KEGG Modules significantly ($p < 0.05$) driving the observed functional dissimilarities of enrichment groups (August *versus* February) were evaluated with Similarity Percentages (SIMPER). The enrichment of significant KEGG Modules is displayed as log 2-fold change. Colour indicates the microbial taxa contributing most to the observed function.

Discussion

The contribution of microbiomes to the health of reef holobionts has been the focus of much recent research (Ainsworth and Gates 2016, Bourne et al 2016, Webster and Thomas 2016), and microbial involvement in the perpetuating cycle of reef degradation via the DDAM feedback loop further highlights the central role of microorganisms in coral reef ecosystem health and resilience (Barott and Rohwer 2012, Haas et al 2016). However, the function of individual microorganisms across different coral reef habitats remains poorly understood. Here we identify the functional potential of different members of sponge, macroalgae and seawater microbiomes using genome-centric metagenomics. We show that switching and shuffling of individual taxonomic groups can have implications for the functional potential of a microbiome and hence, the stability of reef holobionts and ecosystems.

Planktonic *Bacteroidota* MAGs$_{95\%ANI}$ (i.e., family *Flavobacteriaceae* and *Cryomorphaceae*; see Appendix D Table S7.2) were significantly enriched during summer, corresponding with peaks in sea surface temperature and the abundance of canopy-forming brown algae on inshore reefs of the GBR (Schaffelke and Klumpp 1997, Walther et al 2013). Recent 16S rRNA gene based studies have reported similar increases in *Flavobacteriaceae* and *Cryomorphaceae* in algae-dominated reefs (Haas et al 2016), and in inshore-reefs of the GBR, particularly when sea-surface temperatures are high (Glasl et al 2019a). Marine *Bacteroidota* are known to degrade algae-derived polysaccharides via a unique machinery referred to as Polysaccharide Utilization Loci (PULs, Grondin et al 2017) and are major responders to phytoplankton blooms in temperate waters (Krüger et al 2019). The summer-enriched *Bacteroidota* MAGs$_{95\%ANI}$ were all equipped with glycoside hydrolase (GH) families and SusD-like genes (Appendix D Table S7.4 and S7.5), revealing their genomic potential to degrade a diverse range of polysaccharides via the PUL machinery. The most abundant GH in the *Bacteroidota* genomes was the GH16 family (Appendix D Table S7.4), which includes enzymes specific for the degradation of marine polysaccharides (Viborg et al 2019). For example, the GH16 contains the enzyme laminarinase which is known to hydrolyse the *ß*-1,3-d-linked main chain of laminarin into glucose and oligosaccharides (Becker et al 2017). The ubiquitous presence of genes encoding for the GH16 family in *Bacteroidota* MAGs$_{95\%ANI}$ suggests that planktonic *Bacteroidota* are capable of degrading macroalgae derived polysaccharides such as laminarin, a common storage *ß*-glucan of brown algae (Rioux et al 2007). Furthermore, the laminarinase enzyme was recently shown to be ubiquitously present in genomes of marine *Bacteroidota* (Krüger et al 2019), hence, increased *Bacteroidota* in the seawater microbiome may be directly linked to increased macroalgal biomass in the reef ecosystem. Elevated seawater temperatures can enhance the exudation of macroalgae-derived polysaccharides (Mühlenbruch et al 2018) which may also be contributing to the

summertime enrichment of *Bacteroidota*.

In addition to their proposed role in degradation of macroalgae-derived polysaccharides on reefs, planktonic *Bacteroidota* MAGs$_{95\%ANI}$ were also enriched in putative virulence and pathogenic marker genes (Figure 7.6 and Figure 7.7). For example, *Bacteroidota* MAGs$_{95\%ANI}$ have genomic potential to biosynthesise polyketide sugars (dTDP-L-rhamnose biosynthesis) and KDO2-lipid A biosynthesis (Raetz pathway). Polyketide sugars are integrated into the Lipopolysaccharide (LPS) layer of gram-negative bacteria and can help pathogens escape host detection (Lerouge and Vanderleyden 2006). KDO2-lipid A is an essential component of the LPS layer, which can stimulate a host immune response and modulate virulence (Wang et al 2015). Furthermore, the Lipoprotein releasing system and the Twin-arginine translocation (Tat) system (releasing system of folded-proteins) were enriched in summer-elevated *Bacteroidota* MAGs$_{95\%ANI}$. Lipoproteins play key roles in adhesion to host cells, modulation of inflammatory processes and translocation of virulence factors into host cells and can be released via the Tat system (Kovacs-Simon et al 2011). The genomic potential to biosynthesise Phosphatidylethanolamine (PE), an unsaturated fatty acid, was also enriched in *Bacteroidota* MAGs$_{95\%ANI}$. This may allow members of the phylum *Bacteroidota* to tolerate higher temperatures as the increase of unsaturated fatty acids in the LPS significantly contributes to membrane fluidity (Dowhan and Bogdanov 2002).

In contrast to *Bacteroidota*-driven processes during summer, *Alphaproteobacteria* and *Thermoplasmata* MAGs$_{95\%ANI}$ were enriched in reef waters during winter (Figure 7.3 and Figure 7.7) when sea-surface temperatures are low and macroalgae essentially disappears from inshore GBR reefs (Schaffelke and Klumpp 1997). An increase in the alphaproteobacterial family *Pelagibacteraceae* has previously been described for inshore GBR waters during winter (Angly et al 2016, Glasl et al 2019a) and is also more generally associated with increased coral cover (Haas et al 2016). The archaeal *Thermoplasmata* (Marine Group II) have previously been reported as abundant members of the planktonic microbial community of the GBR, with increasing abundances in off-shore reef locations and in reefs with high coral cover (Angly et al 2016). Collectively, our findings support previous studies reporting increased copiotrophic microorganisms and virulence factors on reefs with high algal cover (Haas et al 2016) and with predictions based on the DDAM feedback loop (Barott and Rohwer 2012). Hence, we propose that increased *Bacteroidota* to *Alphaproteobacteria* ratios in reef waters may act as an indicator of enhanced macroalgal growth and the onset of microbialisation in coral reefs. However, in contrast to the previously described shift in central carbohydrate metabolism (i.e., Embden-Meyerhof pathway, Entner-Doudoroff pathway, and pentose phosphate pathway) between coral and algae dominated reefs (Haas et al 2016), the potential to metabolise carbohydrates remained relatively stable between *Alphaprotebacteria* and *Bacteroidota* (Figure S7.4). The

only exception being the Entner-Doudoroff pathway which was more prominent in the winter-enriched Alphaproteobacteria compared to summer-enriched *Bacteroidota* genomes (Figure 7.6). Given the importance of microbially mediated carbohydrate metabolism in coral reefs, identifying changes in the central metabolic pathways of *Alphaproteobacteria* and *Bacteroidota* using sensitive transcriptome/proteomic approaches is warranted.

Sargassum spp., a canopy-forming brown algae, undergoes an annual cycle of growth, reproduction and senescence (Lefèvre and Bellwood 2010). In inshore regions of the GBR, *Sargassum* grows rapidly between October and February, followed by a period of senescence during which it sheds most of its fronds (Lefèvre and Bellwood 2010). During summer, Firmicutes dominate the macroalgal biofilm (up to 91.4 % of the microbiome), having the genomic capacity to generate a hostile environment via production of antibiotics (i.e. Bacilysin) which may hinder opportunistic and biofouling microbes from colonising the macroalgae's surface (Figure 7.2 and Figure 7.7). The functional repertoire associated with *Firmicutes* $MAGs_{95\%ANI}$ (i.e., antibiotic resistance, secondary metabolite production, chemotaxis, multicellular behaviour control, and sporulation control) further suggests that the biofilm reaches its mature state during summer, as these functions are indicative of late successional biofilms (O'Toole et al 2000). Furthermore, *Firmicutes* $MAGs_{95\%ANI}$ had the potential to take up various saccharides (i.e., cellobiose, galactitol, fructose, mannose and mannitol) via the PTS; with the sugar alcohol mannitol being characteristic for *Sargassum* (Zubia et al 2008). The PTS is a common feature of *Firmicutes* and in addition to its primary metabolic function, it is recognized for its regulatory role in biofilm formation, virulence and nitrogen utilization (Saier 2015). In contrast, *Bacteroidota* predominated in the biofilm during winter (Figure 7.2 and Figure 7.7), when *Sargassum* is reduced to a holdfast with one or two short primary axes (Lefèvre and Bellwood 2010). The shift in biofilm associated microbial taxa may indicate a microbial succession synchronised with the seasonal growth-decay cycle of the host and possibly with the availability of sugars. However, a significant role for seaweed-associated microbes in host morphogenesis has previously been reported (Nakanishi et al 1996, Singh et al 2011, Singh and Reddy 2016) and the *Firmicutes* to *Bacteroidota* ratio may also play a direct role in the growth-decay cycle of *Sargassum*. Additionally, macroalgal surfaces may provide a seed bank for planktonic *Bacteroidota* thriving on algal-exudates (detection of four seawater *Bacteroidota* genomes in macroalgae samples; see Appendix D Table S7.6) as well as potentially antagonist bacterial taxa for corals. *Firmicutes* have recently been shown to significantly increase in corals exposed to short-term stress including elevated temperature and macroalgae-abundance (Glasl et al 2019b). Hence, understanding the functional roles of *Bacteroidota* and *Firmicutes* on coral reefs and assessing their potential to invade carbohydrate-rich niches (e.g. coral mucus) is critical.

Marine sponges are a highly diverse component of coral reefs (Diaz and Rützler 2001) where they provide a vital trophic link between the benthic and pelagic realms by removing dissolved organic matter from the reef seawater, making it available to benthos-dwelling life forms as particulate material (De Goeij et al 2013). The health of a sponge holobiont is underpinned by its microbiome (Pita et al 2018, Webster and Taylor 2012, Webster and Thomas 2016). High microbial abundance (HMA) sponges, such as *C. matthewsi* commonly associate with *Chloroflexota* (Moitinho-Silva et al 2017). The *Chloroflexota* $MAGs_{95\%ANI}$ in this study showed genomic potential to biosynthesise secondary metabolites such as dTDP-L-rhamnose (Figure 7.4), a polyketide sugar and O antigen in the bacterial cell wall, which is hypothesised to help sponge amoebocytes differentiate between symbiont and food bacteria (Burgsdorf et al 2015, Lerouge and Vanderleyden 2006). A reduction in sponge-associated *Chloroflexota* could have adverse consequences for host nutrition (carbohydrate metabolism), B-vitamin availability, detoxification (heavy metal detoxification), waste product removal (urea cycle), and the overall health of the sponge holobiont (Figure 7.4 and Figure 7.7). The observed reduction in *Chloroflexota* during summer may reflect a shift in substrate availability and / or a temperature-induced loss of a putative symbiont (Figure 7.2 and Figure 7.7), as has been reported in other sponge species under thermal stress (Fan et al 2013).

Conclusion

Microorganisms have considerable potential to enhance current monitoring tools for reef ecosystem health (Glasl et al 2017, Glasl et al 2019a, Roitman et al 2018), with microbially mediated processes likely underpinning future coral reef trajectories. Our study identified four bacterial groups (*Bacteroidota*, *Alphaproteobacteria*, *Firmicutes* and *Chloroflexota*) whose genomic repertoire (Figure 7.4-7.6) and response to environmental fluctuations (Figure 7.3) suggests a key role in the stability of coral reef ecosystems. We therefore propose that future reef research should employ sensitive metatranscriptome/metaproteome and stable isotope-based approaches to (i) validate macroalgae related shifts in *Bacteroidota* to *Alphaproteobacteria* ratios in reef seawater, (ii) investigate the direct/indirect roles of *Firmicutes* in the health of reef holobionts and (iii) validate the impacts of environmentally driven fluctuations in symbiont abundance on host health.

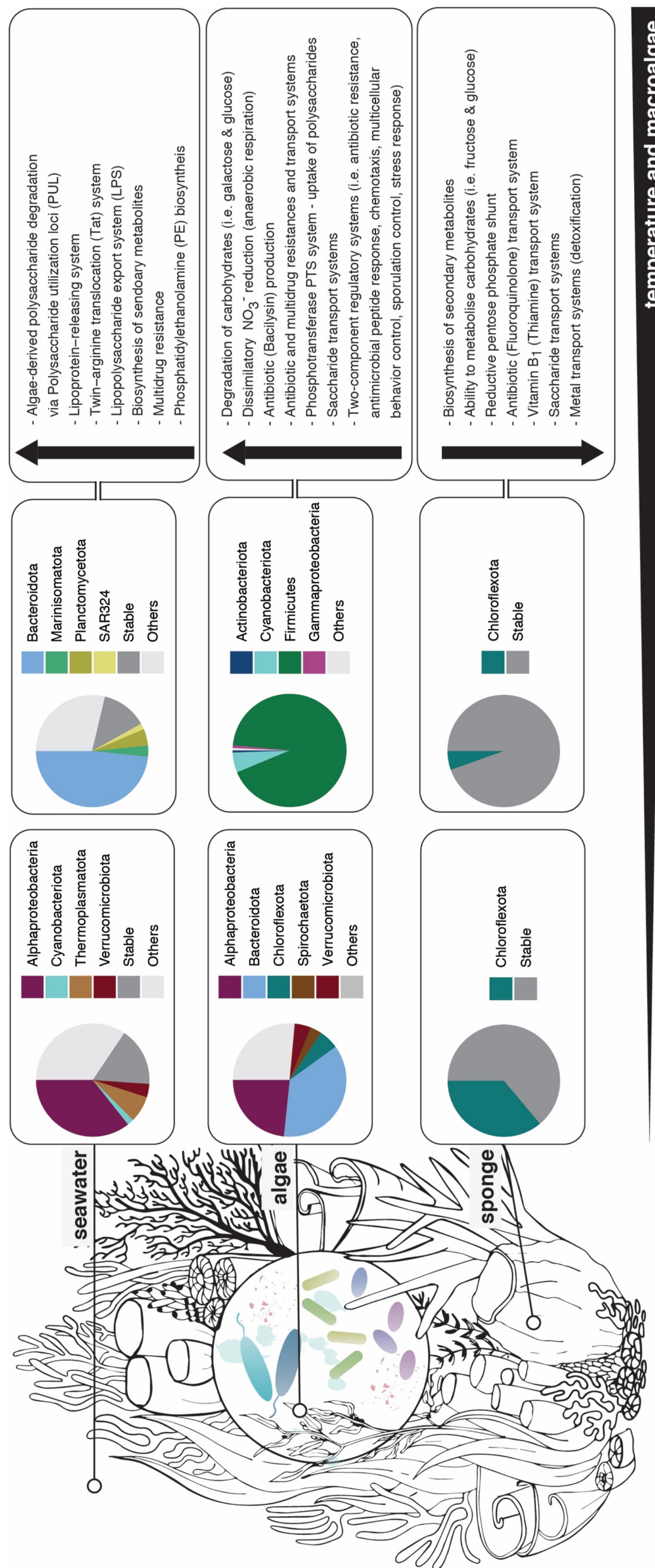

Figure 7.7. Conceptual overview of seasonal changes in coral reef microbiomes. Elevated seawater temperature and increased macroalgal abundance are associated with a shift in the taxonomic composition of seawater, macroalgae and sponge microbiomes and an associated increase in microbial functions associated with opportunistic/pathogenic and copiotrophic process.

Chapter 8

GENERAL DISCUSSION: MICROBES - A NEW TOOL TO MONITOR THE REEF?

Research presented in this thesis provides fundamental insights into the diagnostic potential of microorganisms for coral reef monitoring and highlights how integrated microbial observatories on the Great Barrier Reef (GBR) could enhance our understanding about the role of microbes in coral reef ecosystems. Key findings from this thesis (Box 8.1) will be of substantial interest to a broad cross-section of the scientific community, including researchers and reef managers, and the established comprehensive microbial baselines will allow marine scientists to answer previously intractable questions in coral reef microbial ecology. In this chapter, I discuss recent advances in the field of coral reef microbial monitoring and provide a conceptual framework towards an integrated microbial observatory for the GBR.

Box 8.1. Thesis highlights

- As part of this thesis, the first microbial baseline for selected reef sites on the GBR was established. Compositional (16S rRNA gene sequencing) and functional (metagenome and metatranscriptome) microbial data of multiple host and free-living microbiomes are publicly available at the Bioplatforms Australia data platform under the Australian Microbiome Initiative (Chapter 3).
- Planktonic bacterial community data revealed that the seawater microbiome has a high diagnostic potential to infer environmental perturbations. The seawater microbiome fulfilled key aspects of robust bioindicators (i.e., high habitat specificity, uniform response patterns among sampling replicates, and environmental sensitivity) and was five times more sensitive to changes in the environment than host-associated microbiomes (Chapter 4).
- Coral and sponge tissue microbiomes varied significantly among host genotypes. These observed patterns emphasise the importance of microbiome-by-host genotype-by-environment effects when studying the susceptibility of reef species to environmental disturbances and/or diseases (Chapter 5 and 6).
- Increase in sea-surface temperature and macroalgae abundance significantly correlated with a shift from *Alphaproteobacteria* to *Bacteroidota*-dominated seawater microbiomes. The observed taxonomic shift significantly altered the functional repertoire of the seawater microbiome (e.g., algae-derived polysaccharide degradation, virulence factors, multidrug resistance) and can affect microbial mediated processes in coral reef ecosystems. *Bacteroidota*-dominated seawater microbiomes could act as microbial indicators for the onset of ecosystem degradation (Chapter 7).

Why monitor coral reef microbes?

Recent advances in molecular techniques have unravelled the importance of microbes in coral reef ecosystems. For example, microbes are drivers of biochemical cycles (carbon, nitrogen, sulphur and phosphorus cycle) (Bourne and Webster 2013, Cardini et al 2014, Haas et al 2016, Silveira et al 2017) and form diverse symbiotic associations with reef benthos including corals, sponges and macroalgae (Egan et al 2013, Hentschel et al 2012, Rohwer et al 2002, Thomas et al 2016), where they contribute to host nutrition, nutrient recycling, waste product removal and host defence (Lesser et al 2007, Robbins et al 2019, Slaby et al 2017). Besides the fundamental role of microbes in the health of coral reef ecosystems, microbial communities are able to respond to changing environmental conditions (e.g., Angly et al 2016, Kelly et al 2014, Morrow et al 2015, Zaneveld et al 2016, Ziegler et al 2016). Hence, changes in the taxonomic composition and/or function of microbial communities may provide a sensitive marker for early detection of adverse environmental conditions (Chapter 1 published as Glasl et al 2017, Chapter 2 published as Glasl et al 2018a, Roitman et al 2018). Early detection of adverse environmental conditions and/or the onset of ecosystem degradation is crucial to effectively manage threatened reef systems and mitigate the effects of local and global pressures (Hughes et al 2017). Traditional reef monitoring techniques mainly focus on the end point of ecosystem degradation (i.e., loss of coral cover, high coral mortality), microbial monitoring, in contrast, has a high potential to detect the onset of ecosystem degradation before visual sings of stress occur (Chapter 1 published as Glasl et al 2017). The early detection of environmental stress enhances the ability of reef management agencies to mitigate environmental pressures by implementing, for example, water quality improvement plans, temporal closures of reef sites for commercial and recreational activities, bioremediation programs, and rehabilitation programs (e.g. macroalgae removal). Yet, despite the vast array of monitoring and management efforts implemented via the Reef 2050 Long-Term Sustainability Plan for the GBR, microbial dynamics and their mediated processes across the GBR remain largely unassessed. Integrating microbial monitoring into current reef monitoring initiatives has the potential to enhance assessments of long-term changes in coral reef ecosystems and to identify microbial taxa and/or functions that are critical for reef health. Furthermore, identification of key microbial taxa and/or functions underpinning reef health will improve our understanding of microbial mediated processes in coral reefs and contribute to the current debate about the ability of the microbiome to facilitate long-term acclimatisation of reef species under climate change (Webster and Reusch 2017). For example, microbial indicators and microbial baselines will underpin microbiome manipulation studies aiming to enhance coral reef resilience by building stress tolerant holobionts (van Oppen et al 2015) and the development of probiotics to enhance resilience during short-term environmental stress

events (e.g. bleaching; Peixoto et al 2017).

Diagnostic potential of reef microbiomes

One of the main objectives of this thesis was to evaluate the diagnostic potential of multiple host-associated and free-living reef microbiomes as sensitive indicators to monitor ecosystem health. The diagnostic value of a microbiome and thus its capacity to accurately reflect environmental perturbations, provides crucial information for ecosystem management initiatives aimed at establishing microbial observatories. Based on the results of studies presented in this thesis, I was able to rate the diagnostic value of host tissue microbiomes, host mucus / biofilm microbiomes, and free-living microbial communities according to key characteristics of optimal microbial indicators: (i) uniformity of community assembly, (ii) sensitivity towards environmental fluctuations, and (iii) ease to sample (Figure 8.1.).

Uniformity of microbial community assembly is an essential characteristic for putative microbial indicators as it increases the ability to link changes in the microbial community to environmental variations (Chapter 1 published as Glasl et al 2017). The uniformity of host-associated and free-living microbial communities in response to temporal changes in the environment was assessed by comparing microbial community similarities of sample replicates within a given time point as proxy (Chapter 4 published as Glasl et al 2019a). In general, free-

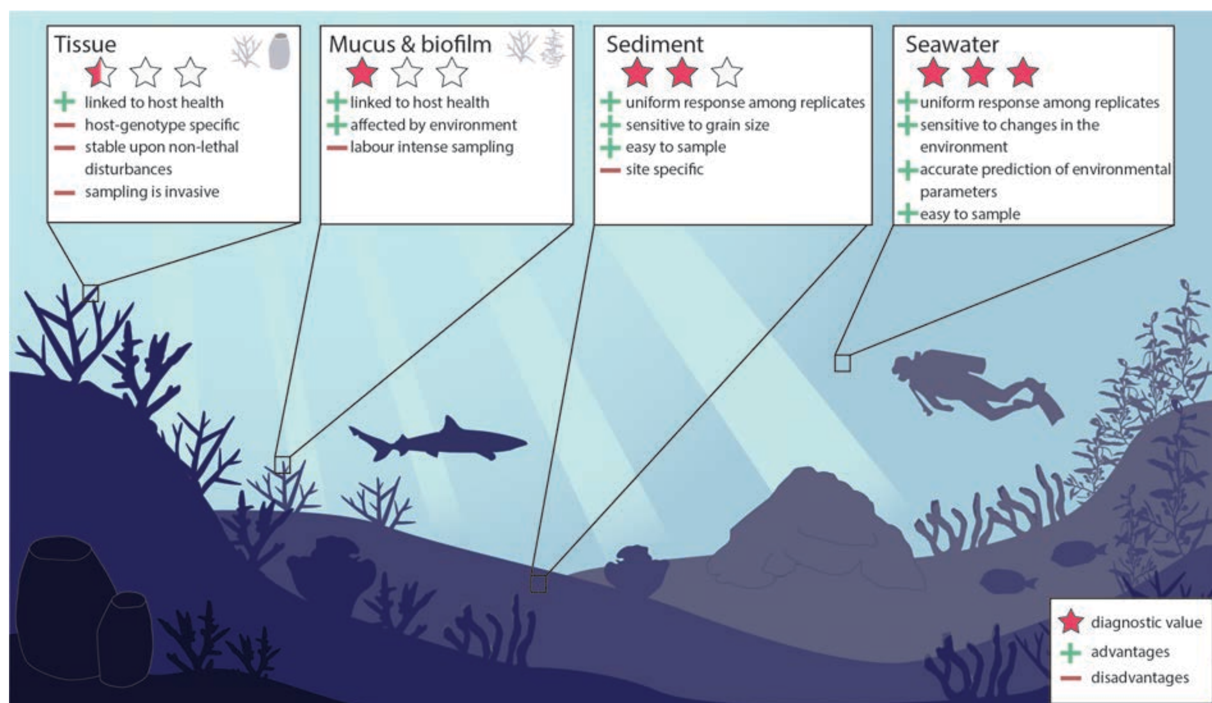

Figure 8.1. Overview of the diagnostic value of various coral reef microbiomes. Microbiomes associated with benthos-dominating macroorganisms such as coral, sponge as macroalgae can be found within the host's tissue and on the host's surface (e.g. coral mucus and macroalgae biofilm). The diagnostic value (indicated as stars) is based on the sum of advantages and disadvantages.

living microbial communities associated with seawater and sediment revealed a higher uniformity in their community assembly pattern than host-associated microbiomes (Chapter 4 published as Glasl et al 2019a). The high compositional similarity of time point replicates of sediment and seawater samples suggests that their community assembly is primarily governed by non-random processes, thus confirming the dominant role of deterministic processes in the assembly of bacterial communities within these environments (Wang et al 2013). Host-associated microbiomes, in contrast, displayed a higher compositional variability of sample replicates within a given time point, suggesting a non-uniform microbial community assembly among host individuals (Chapter 4 published as Glasl et al 2019a). Microbiome instabilities between host individuals may be explained by the 'Anna Karenina' effect, which describes the response of animal microbiomes to stressors that reduce the ability of the holobiont to regulate the microbial community composition and therefore lead to a higher heterogeneity among host individuals (Zaneveld et al 2017). Furthermore, the high variability between sample replicates of host-associated microbiomes might also partially be explained by the high host genotype specificity of coral and sponge microbiomes (Chapter 5 and 6; published as Glasl et al 2018b, Glasl et al 2019b). Hence, fine-scale microbiome variation between host genotypes and non-uniform response patterns of host-associated microbiomes to environmental fluctuations are likely to lower the diagnostic value of host-associated microbiomes.

Environmental sensitivity is a key characteristic of robust indicators (Cooper et al 2009). Hence, the accurate and sensitive response of microbial communities to changes in the environment is of utmost importance for the success of microbial indicator programs. Overall, the seawater microbiome was on average five-times more sensitive to changes in the environment (e.g., temperature and eutrophication) than host associated microbiomes (Chapter 4 published as Glasl et al 2019a). Environmental parameters such as temperature, chlorophyll *a* concentrations, total suspended solids concentrations, and particulate organic carbon concentrations explained 56% of the variation in the seawater microbiome (Chapter 4 published as Glasl et al 2019a). In contrast to the seawater microbiome, microbial communities dwelling in reef sediments varied significantly between sampling sites (Chapter 4 published as Glasl et al 2019a). The high variability of sediment microbiomes among sampling sites suggests that the sediment microbial community is primarily governed by site specific factors (e.g. grain size) rather than by temporal perturbations in the ambient environment (Chapter 4 published as Glasl et al 2019a, Zheng et al 2014). However, bacterial communities of sediments in freshwater stream ecosystems were recently discovered to strongly correlate with the traditional macroinvertebrate biotic index systems, and hence changes in the microbial communities of stream sediments could be used to monitor chronic stressors such as those emerging from urbanisation (Simonin et al 2019).

The stability of the microbiome upon environmental perturbation is vital for the health of the holobiont (reviewed by Pita et al 2018). Changes in the microbiome due to excessive levels of stress have been reported to reduce host health and affect microbiome functions (Botte et al 2019, Fan et al 2013, Glasl et al 2016, Pineda et al 2016). However, microbiomes located in the tissue of their hosts (e.g. corals and sponge) remained highly stable in their taxonomic composition when exposed to non-lethal environmental disturbances (Chapter 5 published as Glasl et al 2018b, Chapter 6 published as Glasl et al 2019b). Similar high resistances in the taxonomic composition of sponge and coral microbiomes have previously been observed under various level of stress including bleaching (Luter et al 2010, Luter et al 2014, Pogoreutz et al 2018, Ziegler et al 2017). Furthermore, coral and sponge microbiomes were also only marginally affected by the environment, which explained on average less than 11% of the observed community variation (Chapter 4 published as Glasl et al 2019a). The low environmental sensitivity of host-associated microbiomes suggests that host factors (e.g., genetics, host health parameters) rather than the environment are shaping the tissue microbiome. However, recent studies showed that the susceptibility of coral tissue-associated microbial communities to reef degradation and anthropogenic impact varies substantially between acroporide, poritide and pocilloporide corals (Beatty et al 2019, Ziegler et al 2019). This highlights the need to further disentangle environmental sensitivities of microbial assemblages between coral taxa. Another critical factor that needs to be considered is the environmental sensitivity of various host compartments. Coral mucus, for example, has been shown to efficiently trap faecal indicator bacteria and human enteric viruses and hence provide an improved medium to detect sewage contamination on coral reefs (Lipp and Griffin 2004). In general, host biofilm-associated microbiomes (e.g., coral mucus and macroalgae biofilm) revealed a slightly higher sensitivity to changes in the environment than host tissue associated microbiomes (Chapter 4 published as Glasl et al 2019a), and the microbiome composition of coral mucus has been shown to be primarily affected by the environment rather than host traits (Pollock et al 2018).

Besides the uniform assembly pattern and environmental sensitivity, non-destructive collection and efficient processing methods are needed for effective integration of microbial monitoring into existing coral reef monitoring programs. The collection of host tissue-associated microbiomes, for example, is labour intensive and poses a certain risk for the health of the sampled organism. A possible alternative for host-tissue collections would be the host-biofilm. Although collections of host-biofilm samples are still labour intensive, they are non-invasive for the host and therefore less destructive in the long term. Free-living microbial communities (seawater and sediment) can be easily collected, without interfering with ecosystem processes and/or the health of reef organisms, consistent with desirable

characteristics for environmental monitoring programs.

Collectively, results revealed that seawater microbial communities have the highest diagnostic potential to infer environmental perturbations, followed by sediment-associated and host-associated microbial communities (Figure 8.1). However, given the importance of microbes to the health of reef holobionts (Ainsworth and Gates 2016, Bourne et al 2016, Pita et al 2018), the establishment of microbial baselines for host-associated microbiomes and the search for host health indicators using more sensitive molecular techniques (e.g. metatranscriptomics, proteomics) are still warranted (Box 8.2).

Box 8.2. Outstanding questions

- Can coral and sponge microbiomes be used as sensitive early warning indicators for the onset of disease and/or bleaching? If so, what are the advantages / disadvantages over more traditional health assessments? Which environmental and/or host-specific factors trigger changes in host-associated microbiomes? Can microbial indicators provide a better understanding of impacts on the host animal and therefore help mangers to make better informed decisions at earlier stages of ecosystem decline?
- How stable are microbial mediated processes across the GBR? How do the microbial communities vary between reef types (i.e. inshore, midshelf, off-shore) in their composition and function? Do microbial indicator systems need to be tailored for each reef type or will a one size-fits-all microbial indicator system provide sufficient insights into microbial mediated reef ecosystem processes?
- Is the highest potential to reveal ecosystem stress given by the occurrence (presence / absence), relative abundance (community level changes), or density (cell counts) of microbial indicators?
- Once microbial indicators for coral reef health are identified, can this information be used in microbiome manipulation projects (i.e. probiotics, assisted evolution) aiming to enhance reef resilience to environment stress and to facilitate long-term acclimatisation of selected keystone reef holobionts to climate change?

Seawater microbiome as putative indicator for reef ecosystem health

Increasing anthropogenic and natural disturbances diminishes the resilience of coral reef ecosystems and fosters the transition from healthy, coral-dominated systems to degraded reefs (De'ath et al 2012, Hughes et al 2007, Hughes et al 2017; Figure 8.2). Shifts in the biomass, composition and function of seawater microbes coincide with the main pressures currently impacting the health of coral reef ecosystems (Silveira et al 2017), such as increasing human impact (Dinsdale et al 2008, Knowles et al 2016), declining water quality (Angly et al

2016, Campbell et al 2015, Chapter 4 published as Glasl et al 2019a), and increasing seawater temperature (Chapter 4 published as Glasl et al 2019a). For example, reefs under anthropogenic pressure are described to have 10 times more microbial cells in the water column than pristine reef sites, and seawater microbial communities are dominated by heterotrophs and potential pathogens (Dinsdale et al 2008). Pristine reefs with little human impact, in contrast, are largely dominated by autotrophic microbial communities (Dinsdale et al 2008). Seasonal weather events and riverine floodwaters are reported to lead to transient changes in the seawater microbiome of nearshore reefs (Angly et al 2016, Campbell et al 2015). Furthermore, the increase in anthropogenic pressures triggers a shift in the trophic structure of coastal marine ecosystems towards higher microbial biomass and energy use, a phenomenon referred to as microbialisation (Jackson et al 2001). Microbialisation has been observed to occur on coral reefs worldwide (Haas et al 2016) and the microbialisation score has been suggested as a powerful metric for assessing the level of human impact a reef system is experiencing (McDole et al 2012). However, seawater microbes not only accurately reflect ecosystem perturbations, but they can ultimately contribute to ecosystem degradation (Silveira et al 2017, Smith et al 2006); i.e. the cumulative effects of overfishing and eutrophication leads to microbialisation of coral reefs, which together act in a positive feedback loop (the DDAM model) to accelerate reef degradation due to the increase of dissolved organic carbon (DOC), coral diseases, algae, and microbes in degraded reef sites (Barott and Rohwer 2012, Haas et al 2011). The proliferation of algae in reef systems enhances the availability of labile DOC, which fuels copiotrophic and potentially pathogenic bacterioplankton communities. These in return increase coral mortality and therefore promote competitive advantages for algae (Barott and Rohwer 2012, Haas et al 2011). However, besides the ability to exacerbate ecosystem degradation, microorganism can also help to maintaining/restore reef health by, for example, enhancing the stress tolerance of corals (Damjanovic et al 2019, Rosado et al 2019), and degrading contaminants after oil spills (Fragoso Ados Santos et al 2015). Hence, monitoring microbial dynamics in the seawater microbiome can provide crucial insights into ecosystem processes underpinning coral reef health and could be informative of phase shifts and ecosystem tipping points.

The occurrence and prevalence of individual microbial taxa in reef seawater samples can also provide a sensitive marker for ecosystem stress. This thesis demonstrated that increasing environmental stressors (e.g., nutrient availability, temperature) and changes in benthic composition (e.g. macroalgae proliferation) were generally correlated with a higher relative abundance of the bacterial phylum *Bacteroidota* in the seawater (Chapter 4 published as Glasl et al 2019a, Chapter 7). Families of the *Bacteroidota* phylum (i.e., *Flavobacteriaceae*,

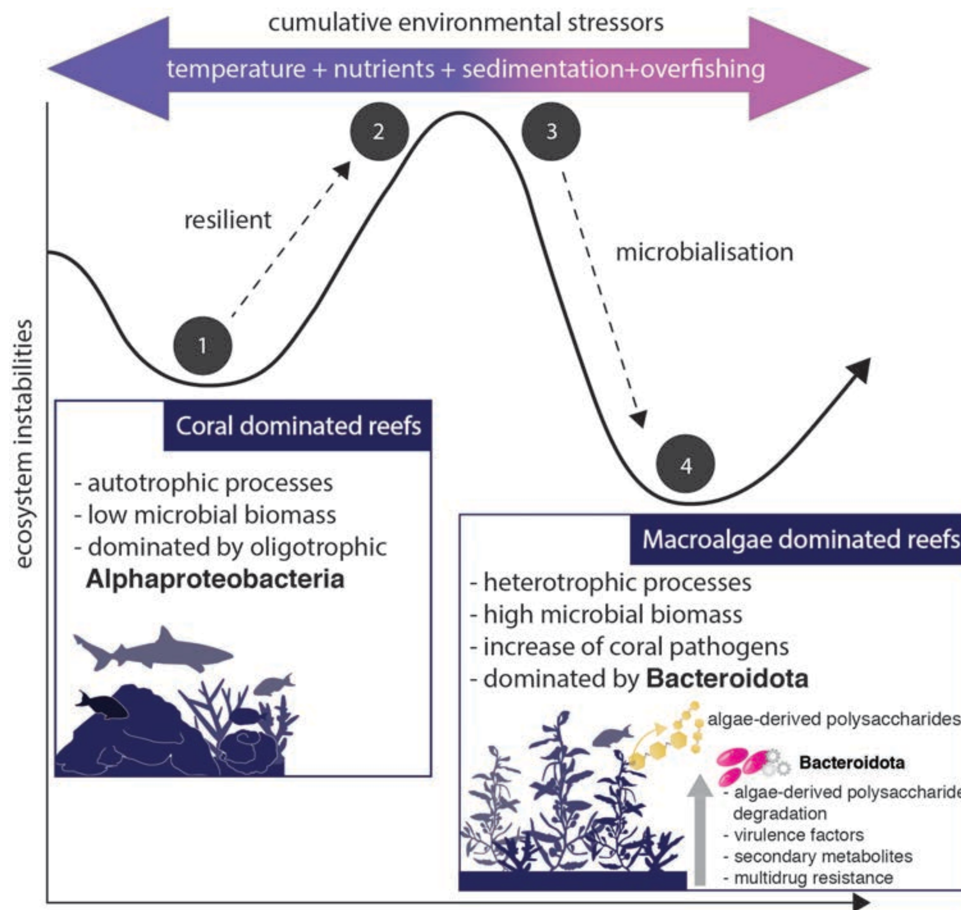

Figure 8.2. Cumulative effects of anthropogenic and natural stressors can shift the stable equilibrium of the reef benthos from a coral-dominated community to degraded reefs, dominated by macroalgae (1-4). As long as cumulative stressors (e.g., elevated sea surface temperature, nutrients, sedimentation, overfishing) remain below the critical threshold (resilience capacity) it is possible for an ecosystem to recover and return to the original stable state (1-2). Once cumulative stressors exceed this threshold, reef ecosystems will experience a loss in coral species richness, reduced resilience, and eventually a shift in the dominant benthic macroorganisms (e.g. macroalgae dominated reef states; 3-4). Seawater microbiomes of healthy, coral-dominated reefs are governed by oligotrophic *Alphaproteobacteria*. The increase of cumulative stressors and the transition from a healthy, coral-dominated reef state to degraded, macroalgal-dominated reef state coincides with an increase of algae-derived polysaccharide degrading *Bacteroidota* in the seawater microbiome.

Cytophagaceae) were previously reported to be enriched in macroalgae-dominated reefs (Haas et al 2016) and in reef sites suffering from poor water quality (Angly et al 2016, Campbell et al 2015). The transition from coral to macroalgae dominance in reef ecosystems has previously been shown to enhance the availability of labile DOC in reef waters (Haas et al 2011), potentially fueling the *Bacteroidota* communities in reef waters. In addition, metagenome sequencing revealed that genomes of the enriched planktonic *Bacteroidota* species are capable of degrading algae-derived polysaccharides such as laminarin (Chapter 7), further emphasizing the potential metabolic link between planktonic *Bacteroidota* and macroalgal proliferation in reef systems (Figure 8.2). Oligotrophic alphaproteobacterial families, such as *Pelagibacteraceae*, were the dominant bacterial taxa in the seawater microbiome during low-stress periods (e.g., lower temperatures, low macroalgae abundances; Chapter 4 published as Glasl et al 2019a, Chapter 7). *Alphaproteobacteria*, were previously reported to be enriched in coral dominated reef systems (Haas et al 2016), suggesting their

prominence in healthy reefs. In this thesis I propose that an increase in the *Bacteroidota*:*Alphaproteobacteria* ratio in seawater microbiomes can be a sensitive marker for the onset of ecosystem instabilities in coral reefs (Figure 8.2). However, the natural spatio-temporal variation in *Bacteroidota* and *Alphaproteobacteria* abundances need to be further assessed, as they are likely to vary among reef locations (e.g. distance to shore) and across temporal scales (e.g. wet *versus* dry season).

Towards integrated microbial observatories for the GBR

Microbial assessments for monitoring ecosystem health are still uncommon, however microbial observatories have the potential to be an important tool for ecosystem health monitoring in the near future (Buttigieg et al 2018, Wegley Kelly et al 2018). Research presented in this thesis provides the first comprehensive microbial assessment of selected reef sites on the GBR (Chapter 3, 4 and 7), and will serve as a foundation for integrated microbial reef monitoring programs. The high diagnostic potential of the free-living microbial communities furthers my recommendation to primarily focus microbial monitoring in coral reef ecosystems on the seawater microbiome. However, microbial baseline datasets for host-associated microbiomes of selected reef species (e.g. with high and low thermo-tolerance) are still crucial as these data will provide valuable insights into microbial contributions to host disease susceptibility and climate resilience.

For robust assessment of seawater-associated microbial communities and their functions across spatio-temporal gradients of the GBR, it will be important to establish regular sample collections at representative reef sites of the almost 3,000 individual reefs of the GBR. This could be achieved, for example, by monthly sample collections along multiple transects from the coast to the GBR shelf edge, covering as many benthic communities (e.g. bioregions sensu Mellin et al 2019) as possible along each transect. Microbial observatories on the GBR may also be established at reef sites of the Long-Term Monitoring Program (LTMP) of the Australian Institute of Marine Science (AIMS). To capture temporal dynamics of planktonic microbes, monthly sampling intervals would be ideal but at the very least, sampling should cover seasonal peaks (wet *versus* dry season). Furthermore, microbial sampling may extend over a representative depth gradient (e.g., 2 m, 10 m, 30 m), to capture depth-related variations in microbial dynamics. In addition to microbial samples, it will be crucial to combine microbial datasets with extensive ecosystem health parameters (e.g., water quality parameters, benthic composition, sedimentation, hydrodynamics), as this will contribute to assessments of microbial dynamics across environmental gradients in coral reef ecosystems and ultimately facilitate the identification of microbial indicator taxa and / or functions.

Standardised protocols allowing streamlined sample collection and processing will be crucial for the establishment of robust microbial monitoring programs. Here I recommend the use of previously established standardised protocols from the Australian Microbiome Initiative (also used in this thesis). Standardised protocols will help to reduce sample biases and further allow comparison of microbial dynamics across datasets. Furthermore, given the current advances in our ability to generate high-throughput data, I recommend to sequence metagenomes in combination with amplicon-based sequencing of the 16S rRNA gene. Large scale metagenome sequencing can establish a solid microbial genome database and therefore provide valuable insights into the functional repertoire of individual microbes. Amplicon datasets, in contrast, will provide a highly streamlined and cost-effective approach to track changes in the presence and relative abundance of individual microbes over space and time. Once a robust microbial genome database is established, 16S rRNA gene sequence reads of microbial taxa of interest can be mapped to genomes to ascertain their functional attributes. Additional aliquots of the raw sample materials and DNA should be kept in biobanks for long-term biomonitoring (Jarman et al 2018), which may warrant a re-analysis in future.

Concluding remarks

Recent advances in our ability to study microbial communities *in situ* has opened a window of opportunity for integrating microbial observatories into established coral reef monitoring initiatives. Research presented in this thesis provides timely information on the diagnostic capacity of reef microbiomes and provides the first comprehensive microbial baseline for selected GBR sites. The key findings of this thesis (Box 8.1) are fundamental for the successful integration of microbial monitoring on the GBR in the near future. Launching microbial observatories in coral reef ecosystems will help to gain new understanding on microbial mediated processes in coral reefs.

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APPENDICES

Appendix A – Supplementary Material for Chapter 4

Table S4.1. Overview on the sample collection during February 2016 till May 2017 at the three sampling sites (Geoffrey Bay, Pioneer Bay, Channel) for each host/habitat. Numbers represent replicates collected per sampling event.

month	Feb-16			Mar-16			Apr-16			May-16			Jun-16			Jul-16			Aug-16			Sep-16		
site	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)
seawater	3			3			3				3	3	3						3					
sediment	3			3			3				3	3	3						3					
A. queenslandica	3			3			3						3						3					
C. matthewsi				3			3				3		3						3					
A. tenuis	3			3			3				3	3	3						3					
A. tenuis mucus	2			2			3				3	3	3						3					
A. millepora							3				3	3	3						3					
A. millepora mucus							3				3	3	3						3					
Sargassum sp.	3			3			3						3						3					

month	Oct-16			Nov-16			Dec-16			Jan-17			Feb-17			Mar-17			Apr-17			May-17		
site	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)	Geoffrey Bay (Magnetic Island)	Pioneer Bay (Orpheus Island)	Channel (Orpheus Island)
seawater	3			3	3	3	3						3			3							3	3
sediment	3			3	3	3	3						3			3							3	3
A. queenslandica	3			3			3						3			3								
C. matthewsi	3			3	3	3	3						3			3							3	3
A. tenuis	3			3	3	3	3						3			3							3	3
A. tenuis mucus	3			3	3	3	3						3			3							3	3
A. millepora	3			3	3	3	3						3			3							3	3
A. millepora mucus	3			3	3	3	3						3			3							3	3
Sargassum sp.	3			3		2	3						3			3							3	

Table S4.2. Statistical output of PERMANOVA (adonis, vegan package) testing the effect of host/habitat on the microbiome composition (10,000 permutations).

```
adonis(formula = d ~ Host, data = df, permutations = 10000, method = "bray")
```

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
Host	8	48.362	6.0453	18.8	0.2879	9.999e-05	***
Residuals	372	119.619	0.3216		0.7121		
Total	380	167.982			1.0000		

Table S4.3. Statistical output of pairwise PERMANOVA (adonis, vegan package) testing compositional variation of microbial communities between host/habitat pairs (999 permutations, Bonferroni adjusted p values).

pairs	F.Model	R2	p.value	p.adjusted
Sediment vs *Sargassum sp.*	13.86609753	0.146164941	0.001	0.036
Sediment vs *C. matthewsi*	34.8432678	0.283640027	0.001	0.036
Sediment vs *A. queenslandica*	27.6842113	0.267005082	0.001	0.036
Sediment vs *A. tenuis*	18.14474956	0.161797584	0.001	0.036
Sediment vs *A. millepora*	17.97854581	0.169643258	0.001	0.036
Sediment vs *A. tenuis* mucus	20.66627379	0.183429105	0.001	0.036
Sediment vs *A. millepora* mucus	17.16713177	0.163236664	0.001	0.036
Sediment vs seawater	73.60303747	0.439150976	0.001	0.036
Sargassum sp. vs *C. matthewsi*	19.72345911	0.208221483	0.001	0.036
Sargassum sp. vs *A. queenslandica*	15.49253476	0.197375901	0.001	0.036
Sargassum sp. vs *A. tenuis*	8.088787516	0.090794675	0.001	0.036
Sargassum sp. vs *A. millepora*	7.968698594	0.096044638	0.001	0.036
Sargassum sp. vs *A. tenuis* mucus	9.579134678	0.108142112	0.001	0.036
Sargassum sp. vs *A. millepora* mucus	7.42494157	0.090081248	0.001	0.036
Sargassum sp. vs seawater	44.1697348	0.352878712	0.001	0.036
C. matthewsi vs *A. queenslandica*	32.90033449	0.319730102	0.001	0.036
C. matthewsi vs *A. tenuis*	21.60797897	0.197138741	0.001	0.036
C. matthewsi vs *A. millepora*	21.15260887	0.205061308	0.001	0.036

C. matthewsi vs *A. tenuis* mucus	26.26867351	0.233980439	0.001	0.036
C. *matthewsi* vs *A. millepora* mucus	21.21690133	0.205556465	0.001	0.036
C. matthewsi vs seawater	89.53183211	0.504314246	0.001	0.036
A. queenslandica vs *A. tenuis*	14.67815749	0.161870928	0.001	0.036
A. queenslandica vs *A. millepora*	13.29072879	0.159570326	0.001	0.036
A. queenslandica vs *A. tenuis* mucus	11.09498399	0.130383525	0.001	0.036
A. queenslandica vs *A. millepora* mucus	9.565676514	0.120223656	0.001	0.036
A. queenslandica vs seawater	26.85720652	0.261111568	0.001	0.036
A. tenuis vs *A. millepora*	4.87949429	0.052535754	0.001	0.036
A. tenuis vs *A. tenuis* mucus	8.518986208	0.084750021	0.001	0.036
A. tenuis vs *A. millepora* mucus	6.727253834	0.071017089	0.001	0.036
A. tenuis vs seawater	42.14157098	0.30954227	0.001	0.036
A. millepora vs *A. tenuis* mucus	8.201091035	0.087059406	0.001	0.036
A. millepora vs *A. millepora* mucus	5.308371529	0.060800258	0.001	0.036
A. millepora vs seawater	39.17007406	0.308013299	0.001	0.036
A. tenuis mucus vs *A. millepora* mucus	1.812593058	0.020641607	0.004	0.144
A. tenuis mucus vs seawater	21.37760951	0.188552304	0.001	0.036
A. millepora mucus vs seawater	22.03462419	0.200251733	0.001	0.036

Table S4.4. Statistical output of the TukeyHSD post hoc test (95% confidence interval) used to compare alpha diversity variances between coral reef habitats.

	diff	**lwr**	**upr**	**p adj**
Sargassum sp.-Sediment	-2.92271459	-3.441838791	-2.4035904	0.0000000
A. queenslandica-Sediment	-4.69996759	-5.243534391	-4.1564008	0.0000000
C. matthewsi-Sediment	-2.95723847	-3.450710862	-2.4637661	0.0000000
A. millepora-Sediment	-4.04136337	-4.534835756	-3.5478910	0.0000000
A. millepora mucus-Sediment	-2.80650334	-3.299975734	-2.3130310	0.0000000
A. tenuis-Sediment	-4.05903506	-4.535774680	-3.5822954	0.0000000
A. tenuis mucus-Sediment	-2.31162759	-2.793521308	-1.8297339	0.0000000
seawater-Sediment	-2.93886053	-3.415600147	-2.4621209	0.0000000
A. queenslandica-*Sargassum* sp.	-1.77725300	-2.358350350	-1.1961556	0.0000000
C. matthewsi-*Sargassum* sp.	-0.03452388	-0.569055699	0.5000079	0.9999999
A. millepora-*Sargassum* sp.	-1.11864877	-1.653180593	-0.5841170	0.0000000
A. millepora mucus-Sargassum sp.	0.11621125	-0.418320572	0.6507431	0.9990218
A. tenuis-*Sargassum* sp.	-1.13632047	-1.655444664	-0.6171963	0.0000000
A. tenuis mucus-*Sargassum* sp.	0.61108700	0.087225549	1.1349484	0.0093714
seawater-*Sargassum* sp.	-0.01614593	-0.535270131	0.5029783	1.0000000
C. matthewsi-*A. queenslandica*	1.74272912	1.184428841	2.3010294	0.0000000
A. millepora-*A. queenslandica*	0.65860422	0.100303946	1.2169045	0.0081029
A. millepora mucus-*A. queenslandica*	1.89346425	1.335163968	2.4517645	0.0000000
A. tenuis-*A. queenslandica*	0.64093253	0.097365731	1.1844993	0.0081513
A. tenuis mucus-*A. queenslandica*	2.38834000	1.840247164	2.9364328	0.0000000
seawater-*A. queenslandica*	1.76110706	1.217540264	2.3046739	0.0000000
A. millepora-*C. matthewsi*	-1.08412489	-1.593780988	-0.5744688	0.0000000
A. millepora mucus-*C. matthewsi*	0.15073513	-0.358920966	0.6603912	0.9915912
A. tenuis-*C. matthewsi*	-1.10179659	-1.595268978	-0.6083242	0.0000000
A. tenuis mucus-*C. matthewsi*	0.64561088	0.147157383	1.1440644	0.0020912
seawater-*C. matthewsi*	0.01837795	-0.475094445	0.5118503	1.0000000
A. millepora mucus-*A. millepora*	1.23486002	0.725203928	1.7445161	0.0000000
A. tenuis-*A. millepora*	-0.01767169	-0.511144083	0.4758007	1.0000000
A. tenuis mucus-*A. millepora*	1.72973577	1.231282278	2.2281893	0.0000000
seawater- *A. millepora*	1.10250284	0.609030450	1.5959752	0.0000000
A. tenuis-*A. millepora* mucus	-1.25253171	-1.746004105	-0.7590593	0.0000000
A. tenuis mucus-*A. millepora* mucus	0.49487575	-0.003577744	0.9933292	0.0533513
seawater-A. millepora mucus	-0.13235718	-0.625829572	0.3611152	0.9956668
A. tenuis mucus-*A. tenuis*	1.74740746	1.265513749	2.2293012	0.0000000
seawater-*A. tenuis*	1.12017453	0.643434911	1.5969142	0.0000000
seawater-*A. tenuis* mucus	-0.62723293	-1.109126644	-0.1453392	0.0019332

Table S4.5. Average alpha diversities based on Shannon Index for the distinct coral reef habitats (N= number of samples, sd= standard deviation, se = standard error, ci= 95% confidence interval).

Habitat	N	Shannon	sd	se	ci
Sediment	48	7.371837	0.1883402	0.02718456	0.05468829
Sargassum sp.	35	4.449122	1.4363004	0.24277908	0.49338645
A. queenslandica	30	2.671869	0.7893556	0.14411595	0.29475021
C. matthewsi	42	4.414598	0.3085990	0.04761785	0.09616621
A. millepora	42	3.330474	0.6596632	0.10178824	0.20556552
A. millepora mucus	42	4.565334	0.8739431	0.13485235	0.27233985
A. tenuis	48	3.312802	0.8167603	0.11788920	0.23716248
A. tenuis mucus	46	5.060209	0.8457770	0.12470301	0.25116476
seawater	48	4.432976	0.2036506	0.02939443	0.05913397

Table S4.6. Average observed zOTU richness for the distinct coral reef habitats (N= number of samples, sd= standard deviation, se = standard error, ci= 95% confidence interval).

Habitat	N	Observed	sd	se	ci
Sediment	48	2174.8125	202.93754	29.291511	58.92692
Sargassum sp.	35	690.2286	391.07958	66.104515	134.34054
A. queenslandica	30	256.3667	173.59435	31.693847	64.82120
C. matthewsi	42	288.9762	67.18358	10.366652	20.93588
A. millepora	42	147.4048	48.60044	7.499211	15.14496
A. millepora mucus	42	323.2381	267.07492	41.210555	83.22641
A. tenuis	48	184.4167	162.22625	23.415342	47.10559
A. tenuis mucus	46	569.6957	363.28941	53.564101	107.88364
seawater	48	585.7083	54.47895	7.863359	15.81904

Table S4.7. Statistical outputs of permutational ANOVAs for dbRDA analyses (anova.cca, vegan package) testing the significance of each predictor variable (10,000 permutations) on the microbiome composition for all habitats individually.

a) Seawater

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.25153	10.0055	9.999e-05	***
TSS	1	0.14231	5.6610	9.999e-05	***
NPOC	1	0.19049	7.5777	9.999e-05	***
Chla	1	0.15292	6.0831	9.999e-05	***
avg_daylight	1	0.29859	11.8776	9.999e-05	***
POC	1	0.27342	10.8763	9.999e-05	***
Season	1	0.11235	4.4693	9.999e-05	***
Sampling_Date	1	0.08958	3.5633	0.0005999	***
Residual	21	0.52792			

b) Sediment

	Df	Variance	F	Pr(>F)	
avg_temp	1	0.2714	1.4859	0.083916	.
TSS	1	0.3062	1.6762	0.059940	.
corse	1	0.6198	3.3929	0.001998	**
sand	1	0.4791	2.6224	0.006993	**
TOC_Sediment	1	0.5556	3.0414	0.001998	**
TON_Sediment	1	0.2299	1.2588	0.201798	
Season	1	0.6750	3.6948	0.000999	***
Residual	34	6.2110			

c) *A. tenuis* mucus

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.5119	1.8817	0.0021	**
TSS	1	0.9276	3.4098	9.999e-05	***
NPOC	1	0.4017	1.4766	0.0287	*
Chla	1	0.3780	1.3895	0.0476	*
avg_daylight	1	0.4955	1.8213	0.0033	**
POC	1	0.4101	1.5076	0.0325	*
Season	1	0.4058	1.4917	0.0270	*
Sampling_Date	1	0.2561	0.9413	0.5629	
Residual	19	5.1689			

d) *A. millepora* mucus

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.5059	1.3576	0.0267973	*
TSS	1	0.6244	1.6755	0.0008999	***
NPOC	1	0.4456	1.1959	0.1076892	
Chla	1	0.5648	1.5156	0.0071993	**
avg_daylight	1	0.4616	1.2386	0.0701930	.
POC	1	0.4020	1.0788	0.2514749	
Sampling_Date	1	0.4628	1.2419	0.0688931	.
Residual	16	5.9623			

e) *Sargassum* sp. biofilm

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.8476	2.5646	0.000200	***
TSS	1	0.4187	1.2668	0.115788	
NPOC	1	0.5595	1.6929	0.008299	**
Chla	1	0.6494	1.9650	0.002100	**
avg_daylight	1	0.4930	1.4918	0.033797	*
POC	1	0.8226	2.4890	9.999e-05	***
Season	1	0.5648	1.7089	0.006899	**
Sampling_Date	1	0.4994	1.5110	0.025697	*
Residual	21	6.9403			

f) *A. tenuis* tissue

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.5692	1.6364	0.0374	*
TSS	1	1.0185	2.9279	9.999e-05	***
NPOC	1	0.4732	1.3604	0.1044	
Chla	1	0.6047	1.7383	0.0232	*
avg_daylight	1	0.3951	1.1360	0.2606	
POC	1	0.5607	1.6120	0.0380	*
Season	1	0.8261	2.3750	0.0020	**
Sampling_Date	1	0.2545	0.7317	0.8601	
Residual	21	7.3048			

g) *A. millepora* tissue

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.4590	1.1638	0.19118	
TSS	1	0.4239	1.0748	0.31617	
NPOC	1	0.5043	1.2788	0.07799	.
Chla	1	0.3766	0.9550	0.53615	
avg_daylight	1	0.5219	1.3234	0.07139	.
POC	1	0.3442	0.8728	0.72153	
Season	1	0.4623	1.1722	0.17158	
Residual	16	6.3102			

h) *C. matthewsi*

	Df	SumOfSqs	F	Pr(>F)	
avg_temp	1	0.23898	1.5206	0.0380	*
TSS	1	0.26510	1.6868	0.0124	*
NPOC	1	0.20436	1.3003	0.1221	
Chla	1	0.20464	1.3021	0.1220	
avg_daylight	1	0.40672	2.5879	9.999e-05	***
POC	1	0.19280	1.2267	0.1721	
Season	1	0.16873	1.0736	0.3488	
Sampling_Date	1	0.15981	1.0169	0.4338	
Residual	18	2.82895			

i) *A. queenslandica*

```
              Df  SumOfSqs      F     Pr(>F)
avg_temp       1    0.3385 1.7979     0.1204
TSS            1    0.1998 1.0609     0.3259
NPOC           1    0.7094 3.7676     0.0109 *
Chla           1    0.1839 0.9768     0.3689
avg_daylight   1    0.2428 1.2892     0.2370
POC            1    1.3842 7.3512     0.0003 ***
Season         1    0.1909 1.0137     0.3422
Sampling Date  1    0.1044 0.5542     0.7404
Residual      21    3.9543
```

Table S4.8. Host-associated microbiomes and their sensitivity (% explained, Variation Partitioning Analysis, var.par, vegan package) to environmental parameters (permutational ANOVAs for dbRDA analyses (anova.cca, vegan package).

microbiome	n	% explained	environmental parameters (p-values: <0.05*, <0.01** and <0.001***)
A. tenuis mucus	28	17	temperature**, daylight**, TSS***, NPOC*, Chla* and POC*
A. millepora mucus	24	6	temperature*, TSS*** and Chla**
Sargassum sp. biofilm	30	14	temperature***, NPOC**, Chla**, daylight* and POC***
A. tenuis tissue	30	14	Temperature*, TSS*** and Chla*
A. millepora tissue	24	0	n.s.
C. matthewsi	27	6	Temperature*, TSS* and daylight***
A. queenslandica	30	19	NPOC* and POC***
seawater	30	56	temperature***, daylight***, TSS***, NPOC***, Chla*** and POC***
sediment	30	3	n.s.

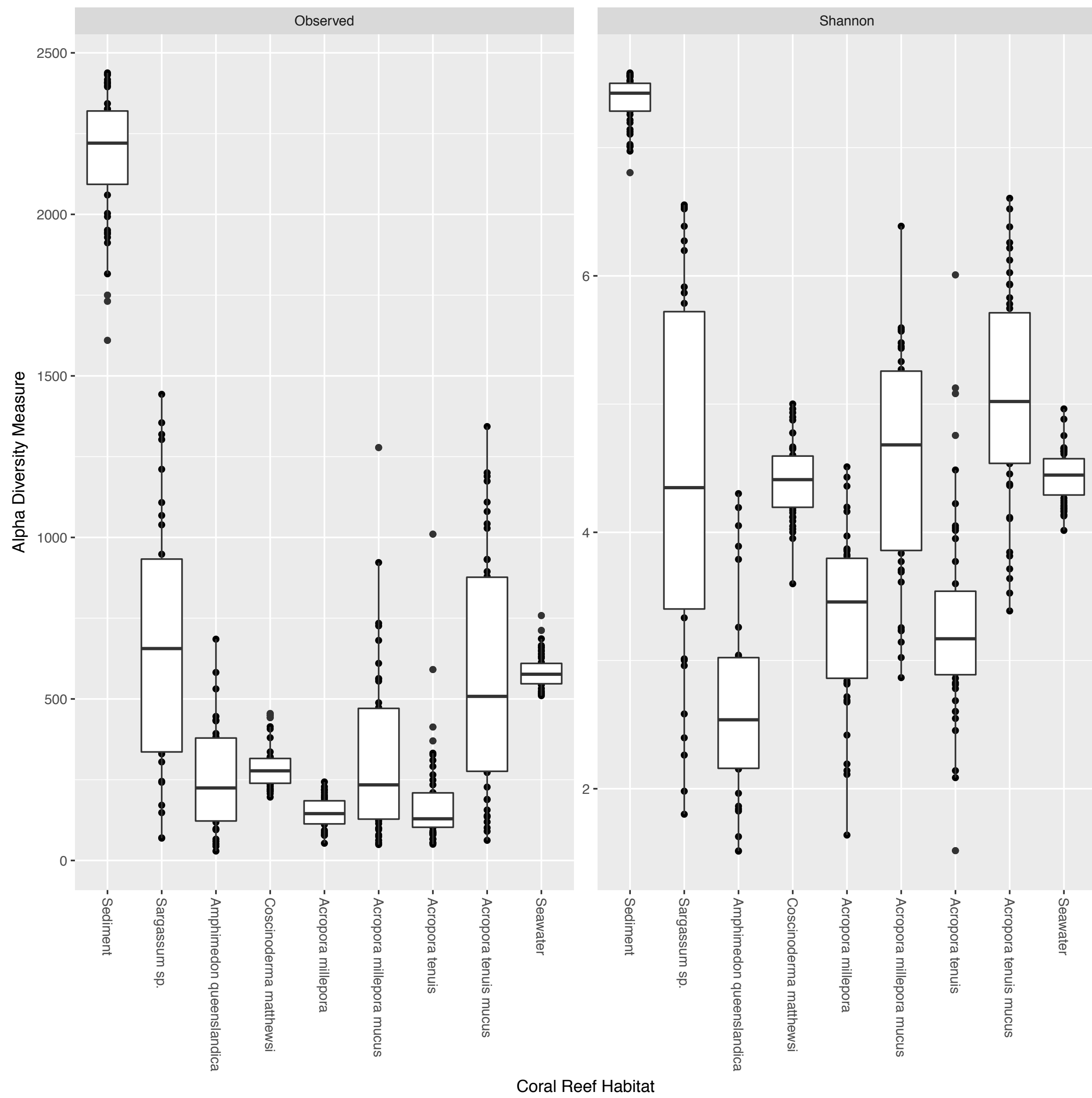


Figure S4.1. Observed zOTU richness (left) and alpha diversity (right) of microbial communities associated with the distinct coral reef habitats. Alpha diversity was calculated with the Shannon Index (richness and evenness).

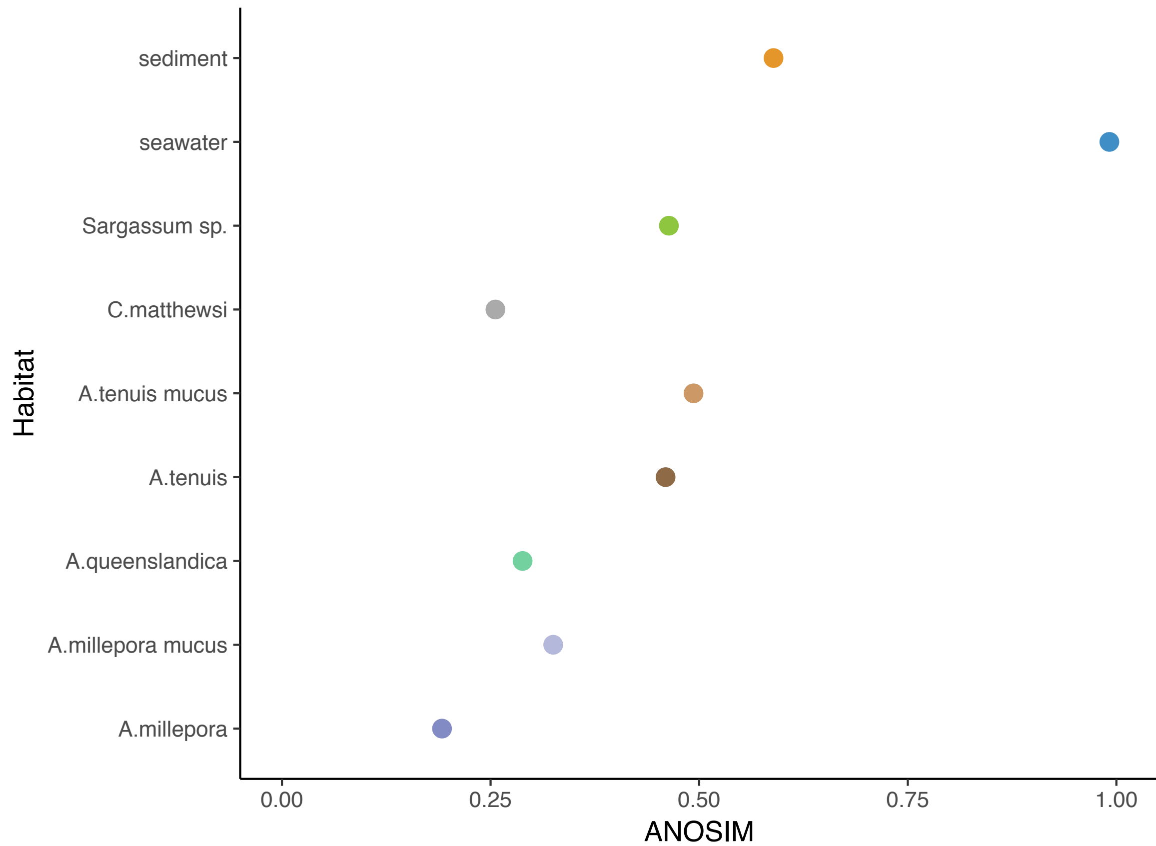


Figure S4.2. Within and between time point similarities of microbial community composition using the ANOSIM R value as proxy (R = 0 indicates an even distribution of high and low dissimilarity ranks within and between time points and R = 1 indicates higher within than between sampling time points similarities).

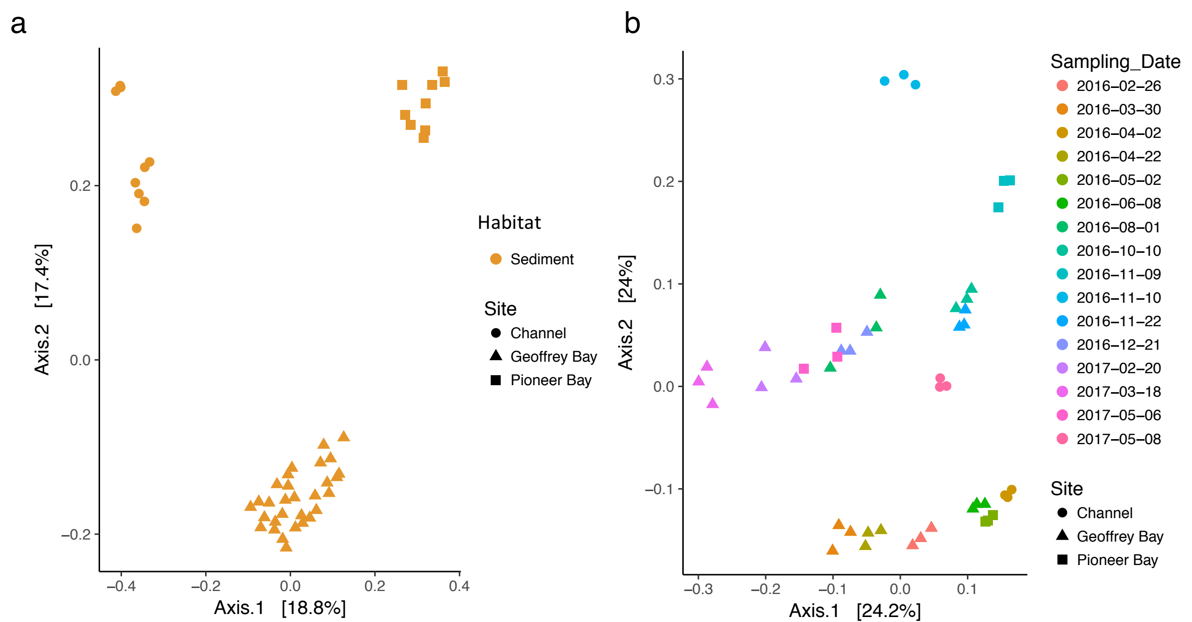


Figure S4.3. PCoA plots for a) sediment and b) seawater illustrating compositional variations between samples collected at different sampling locations. The total variance (in percent) explained by each axis is indicated in parentheses.

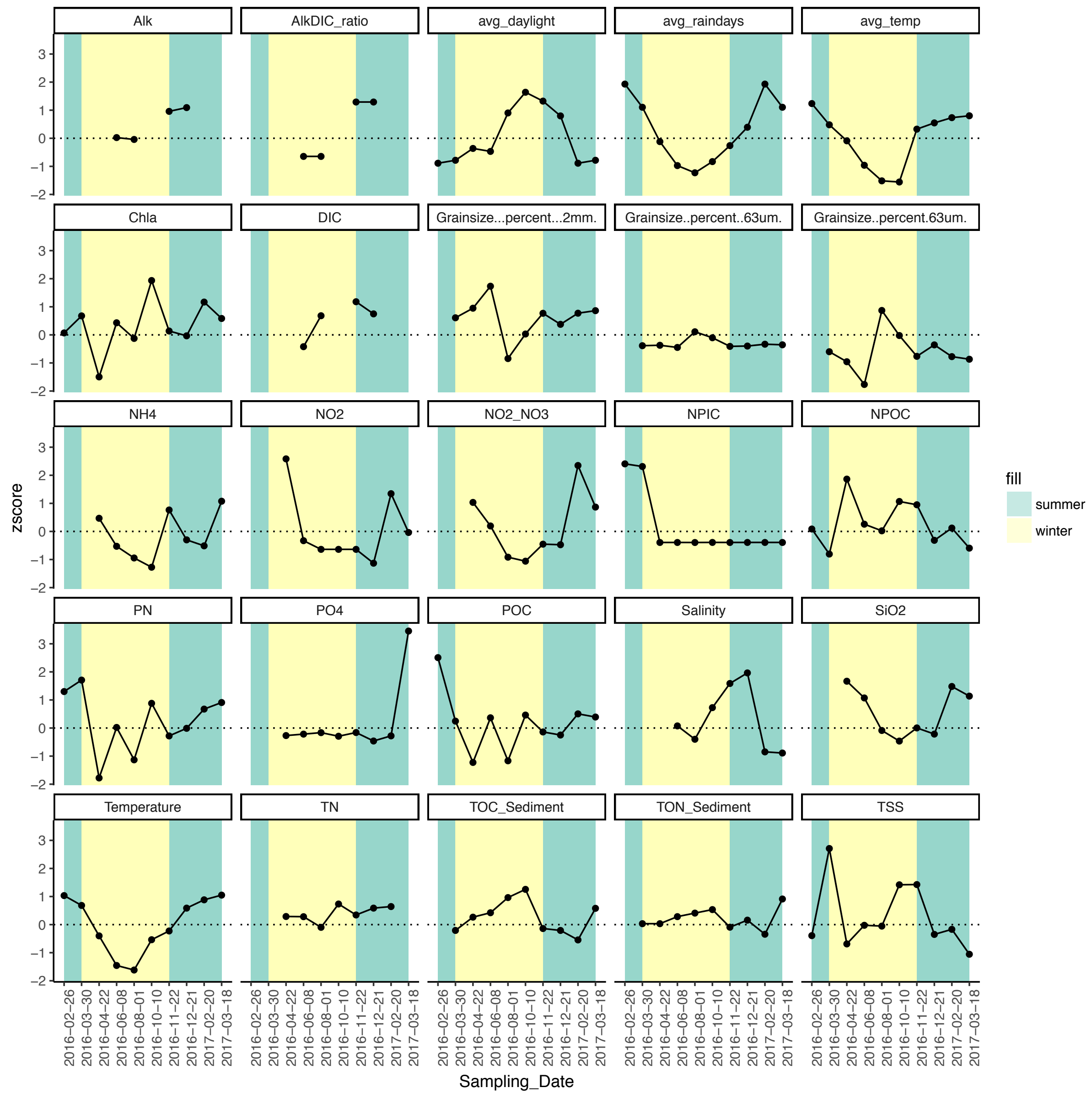


Figure S4.4. Environmental variability at the Geoffrey Bay (Magnetic Island) sampling location. Environmental metadata were standardized using the z-score standardisation method.

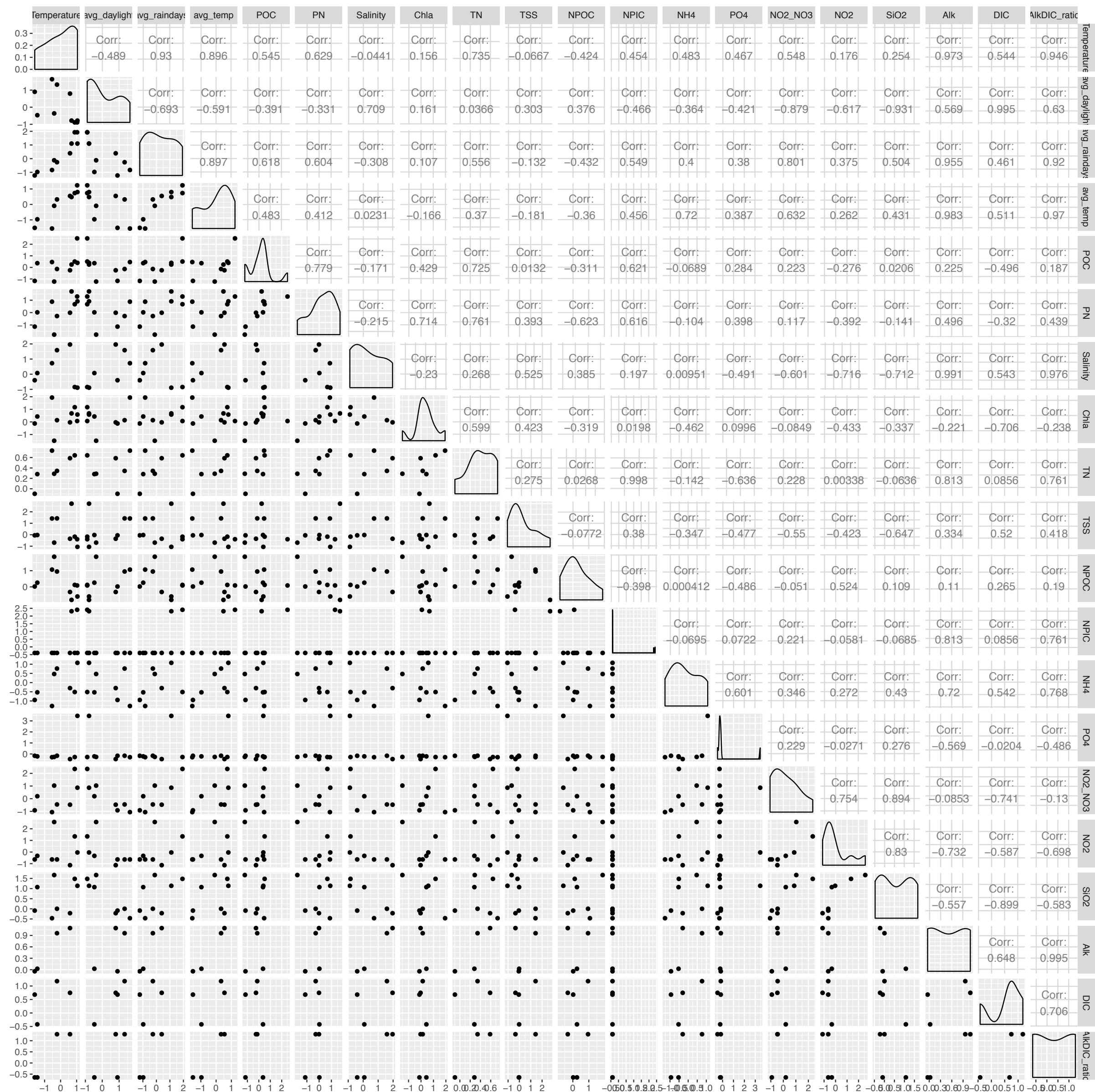


Figure S4.5. Collinearity of environmental metadata collected at Geoffrey Bay (Magnetic Island) calculated with Pearson correlation (collinearity threshold: > 0.7 or < -0.7).

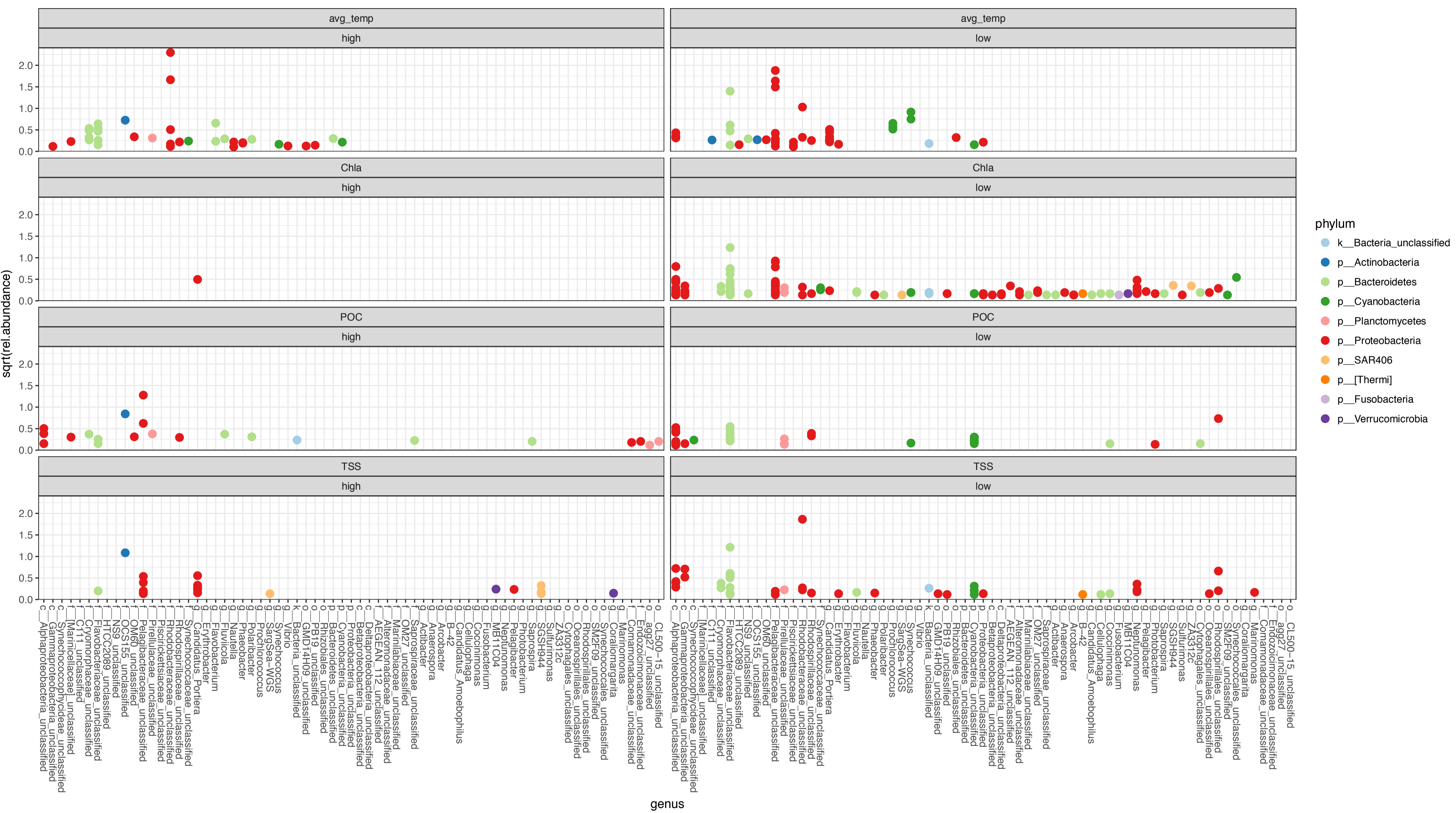


Figure S4.6. Microbial indicator taxa, calculated with the Indicator Value analysis, for high and low temperature, Chla, POC and TSS concentrations. Each dot represents a unique zOTU.

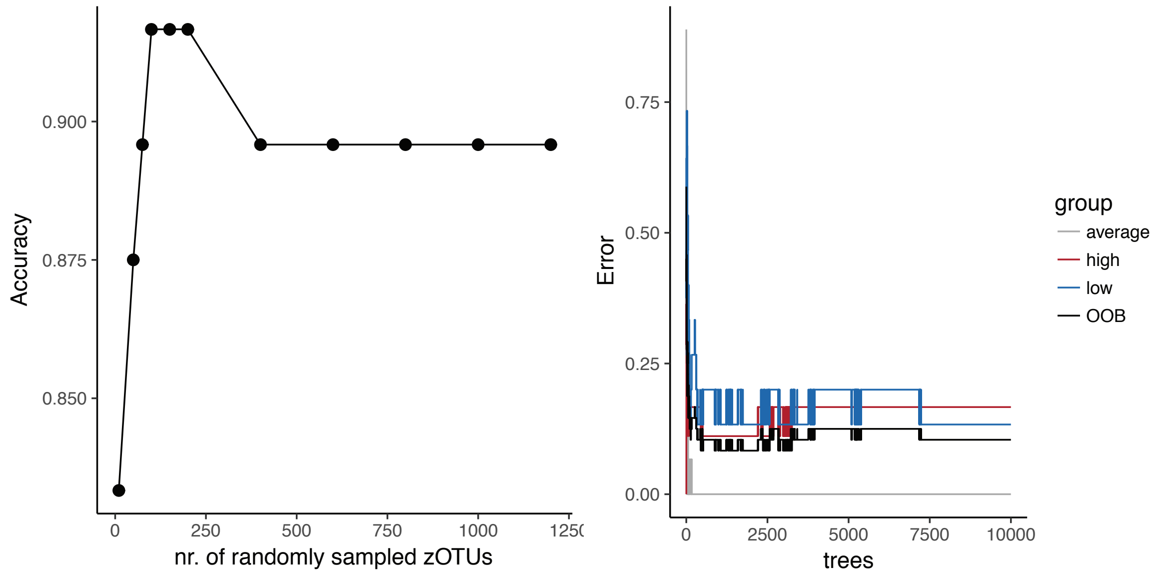


Figure S4.7. Classification of seawater temperature based on Random Forest machine learning. Highest accuracy was achieved with 100 randomly sampled zOTUs (left). Out-of-bag (OOB) error with 10,000 trees (right).

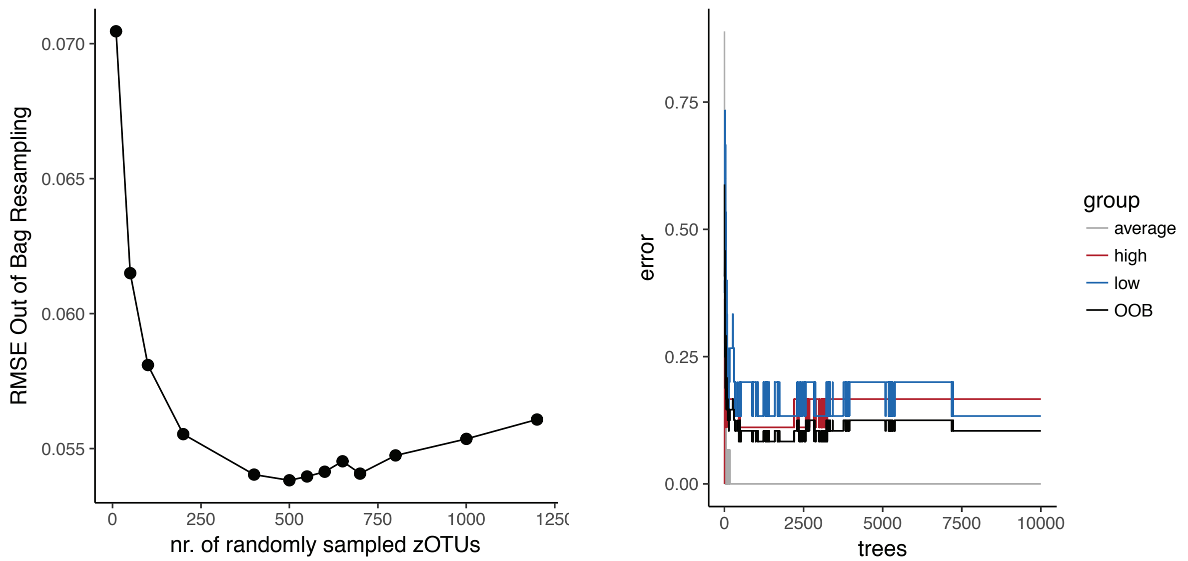


Figure S4.8. Random Forest machine learning seawater temperature regression. RMSE (Root Mean Square Error), based on Out-of-Bag resampling, was lowest with 400 randomly sampled zOTUs (left). Out-of-bag (OOB) error with 10,000 trees (right).

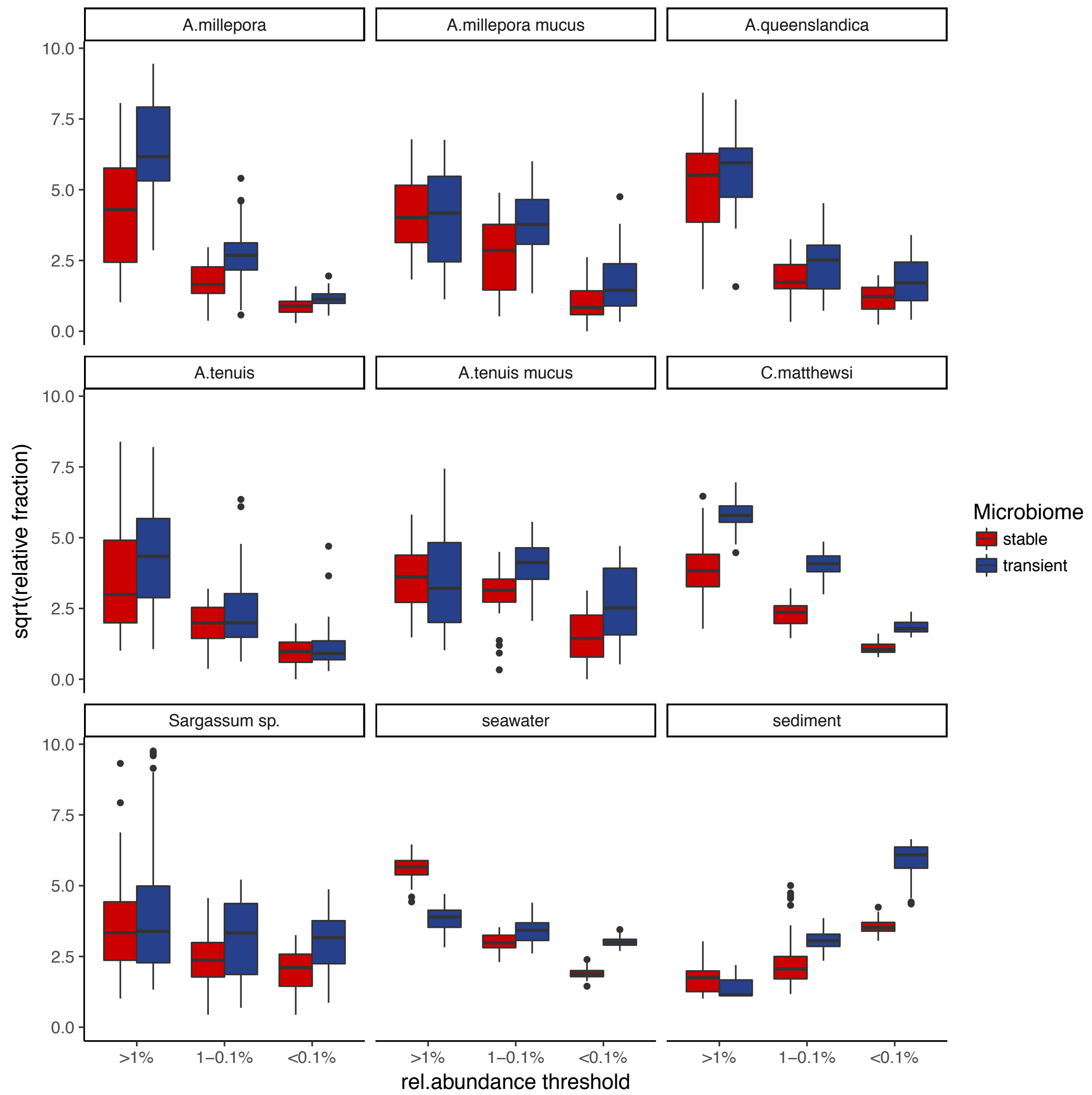


Figure S4.9. Stable *versus* transient microbiomes based on different abundance thresholds. Microbiomes were grouped based on their average relative abundances (>1%, 1-0.1% and <0.1%). The fractional contribution of stable (present in >80% of the samples) and transient (<50% of the samples) microbiomes are represented as boxplots.

Appendix B – Supplementary Material for Chapter 5

Table S5.1. Statistical output of PERMANOVA (adonis2, vegan package) testing the effect of treatment and sampling time point on the photopigment composition within each host species (10 000 permutations).

```
adonis2(formula = d ~ Treatment * SamplingTimepoint, data = df, permutations = perm, method = "bray")
```

	Df	**SumOfSqs**	**F**	**Pr(>F)**
Treatment	1	0.0173	0.0987	0.8619
SamplingTimepoint	2	0.0931	0.2657	0.7179
Treatment:SamplingTimepoint	2	0.0650	0.1857	0.8379
Residual	100	17.5122		

Table S5.2. Overview of microbiome diversity metrics (mean ± standard deviation) for each sponge species for both treatments (control and disturbance) over time. Richness, evenness and Shannon Index were calculated based on a non-rarefied feature table excluding singletons, chloroplast and mitochondria derived reads.

Host	**Sampling day**	**Richness**		**Evenness**		**Shannon Index**	
		Control	Disturbance	Control	Disturbance	Control	Disturbance
AQ	1	362 (±137)	257 (±105)	0.459 (±0.133)	0.476 (± 0.144)	2.71 (± 0.95)	3.55 (± 0.95)
	11	309 (±125)	262 (±68)	0.539 (±0.051)	0.638 (± 0.045)	3.07 (± 0.42)	3.55 (± 0.39)
	17	307 (±110)	282 (±38)	0.532 (±0.158)	0.505 (± 0.139)	3.02 (± 0.89)	2.84 (± 0.73)
CO	1	329 (±12)	261 (±53)	0.848 (±0.012)	0.855 (± 0.010)	4.92 (± 0.10)	4.75 (± 0.20)
	11	263 (±17)	215 (±14)	0.836 (±0.029)	0.863 (± 0.005)	4.66 (± 0.19)	4.64 (± 0.08)
	17	234 (±55)	201 (±12)	0.856 (±0.007)	0.856 (± 0.014)	4.65 (± 0.23)	4.54 (± 0.12)
CY	1	99 (±3)	94 (±6)	0.699 (±0.044)	0.709 (± 0.024)	3.21 (± 0.21)	3.22 (± 0.08)
	11	98 (±7)	82 (±15)	0.665 (± 0.024)	0.664 (± 0.073)	3.05 (± 0.15)	2.93 (± 0.44)
	17	94 (±9)	92 (±11)	0.717 (± 0.012)	0.700 (± 0.031)	3.26 (± 0.02)	3.17 (± 0.22)
IB	1	29 (±3)	51 (±44)	0.382 (± 0.061)	0.362 (± 0.091)	1.29 (± 0.24)	1.38 (± 0.69)
	11	55 (±28)	102 (±99)	0.410 (± 0.043)	0.376 (± 0.081)	1.62 (± 0.35)	1.67 (± 0.74)
	17	87 (±108)	72 (±49)	0.438 (± 0.069)	0.334 (± 0.060)	1.75 (± 0.90)	1.40 (± 0.46)
IR	1	112 (±19)	121 (±10)	0.768 (± 0.018)	0.760 (± 0.018)	3.61 (± 0.07)	3.64 (± 0.11)
	11	119 (±5)	121 (±11)	0.768 (± 0.031)	0.792 (± 0.009)	3.67 (± 0.12)	3.79 (± 0.02)
	17	110 (±11)	116 (± 9)	0.772 (± 0.014)	0.795 (± 0.008)	3.63 (± 0.05)	3.78 (± 0.09)
ST	1	123 (±20)	89 (± 11)	0.683(± 0.032)	0.574 (± 0.105)	3.28 (± 0.20)	2.56 (± 0.41)
	11	139 (±73)	59 (± 19)	0.622 (± 0.188)	0.559 (± 0.085)	3.07 (± 1.27)	2.28 (± 0.49)
	17	95 (±39)	63 (± 25)	0.504 (± 0.114)	0.538 (± 0.033)	2.26 (± 0.44)	2.20 (± 0.37)

Table S5.3. Statistical output of ANOVA using to test the differences in alpha diversity (Shannon Index) between treatments, sampling time points and host species.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
SamplingTimepoint	2	0.28	0.140	0.589	0.558	
Treatment	1	0.26	0.259	1.090	0.300	
Host	5	101.48	20.297	85.356	<2e-16	***
SamplingTimepoint:Treatment	2	0.04	0.020	0.083	0.921	
SamplingTimepoint:Host	10	3.15	0.315	1.324	0.234	
Treatment:Host	5	1.13	0.226	0.950	0.454	
SamplingTimepoint:Treatment:Host	10	1.06	0.106	0.445	0.919	
Residuals	72	17.12	0.238			

Table S5.4. Statistical output of the TukeyHSD post hoc test (95% confidence interval) used to compare group dispersion variances between host species and treatment groups. Treatment groups within a host species are highlighted in grey.

Host_Treatment	**diff**	**lwr**	**upr**	**p adj**
AQ_Treatment-AQ_Control	0.036448345	-0.057054000	0.1299506905	0.9764472
CO_Control-AQ_Control	-0.145971784	-0.239474129	-0.0524694384	0.0000615

CO_Treatment-AQ_Control	-0.163788931	-0.257291276	-0.0702865853	0.0000040
CY_Control-AQ_Control	-0.258198351	-0.351700696	-0.1646960058	0.0000000
CY_Treatment-AQ_Control	-0.196917228	-0.290419573	-0.1034148823	0.0000000
IB_Control-AQ_Control	-0.311864954	-0.405367299	-0.2183626084	0.0000000
IB_Treatment-AQ_Control	-0.382482895	-0.475985241	-0.2889805499	0.0000000
IR_Control-AQ_Control	-0.109206085	-0.202708430	-0.0157037395	0.0088613
IR_Treatment-AQ_Control	-0.193376531	-0.286878876	-0.0998741853	0.0000000
ST_Control-AQ_Control	-0.062353112	-0.155855457	0.0311492338	0.5298100
ST_Treatment-AQ_Control	-0.013147022	-0.106649368	0.0803553229	0.9999984
CO_Control-AQ_Treatment	-0.182420129	-0.275922474	-0.0889177836	0.0000002
CO_Treatment-AQ_Treatment	-0.200237276	-0.293739621	-0.1067349305	0.0000000
CY_Control-AQ_Treatment	-0.294646696	-0.388149042	-0.2011443510	0.0000000
CY_Treatment-AQ_Treatment	-0.233365573	-0.326867918	-0.1398632274	0.0000000
IB_Control-AQ_Treatment	-0.348313299	-0.441815644	-0.2548109536	0.0000000
IB_Treatment-AQ_Treatment	-0.418931240	-0.512433586	-0.3254288951	0.0000000
IR_Control-AQ_Treatment	-0.145654430	-0.239156775	-0.0521520847	0.0000645
IR_Treatment-AQ_Treatment	-0.229824876	-0.323327221	-0.1363225305	0.0000000
ST_Control-AQ_Treatment	-0.098801457	-0.192303802	-0.0052991114	0.0288267
ST_Treatment-AQ_Treatment	-0.049595368	-0.143097713	0.0439069777	0.8261911
CO_Treatment-CO_Control	-0.017817147	-0.111319492	0.0756851985	0.9999635
CY_Control-CO_Control	-0.112226567	-0.205728913	-0.0187242220	0.0061545
CY_Treatment-CO_Control	-0.050945444	-0.144447789	0.0425569015	0.8000257
IB_Control-CO_Control	-0.165893170	-0.259395515	-0.0723908246	0.0000029
IB_Treatment-CO_Control	-0.236511111	-0.330013457	-0.1430087661	0.0000000
IR_Control-CO_Control	0.036765699	-0.056736646	0.1302680443	0.9748654
IR_Treatment-CO_Control	-0.047404747	-0.140907092	0.0460975984	0.8645319
ST_Control-CO_Control	0.083618672	-0.009883673	0.1771210176	0.1259268
ST_Treatment-CO_Control	0.132824761	0.039322416	0.2263271067	0.0004103
CY_Control-CO_Treatment	-0.094409420	-0.187911766	-0.0009070752	0.0456199
CY_Treatment-CO_Treatment	-0.033128297	-0.126630642	0.0603740484	0.9888108
IB_Control-CO_Treatment	-0.148076023	-0.241578368	-0.0545736777	0.0000450
IB_Treatment-CO_Treatment	-0.218693965	-0.312196310	-0.1251916192	0.0000000
IR_Control-CO_Treatment	0.054582846	-0.038919500	0.1480851911	0.7212640
IR_Treatment-CO_Treatment	-0.029587600	-0.123089945	0.0639147453	0.9956340
ST_Control-CO_Treatment	0.101435819	0.007933474	0.1949381644	0.0216375
ST_Treatment-CO_Treatment	0.150641908	0.057139563	0.2441442536	0.0000306
CY_Treatment-CY_Control	0.061281124	-0.032221222	0.1547834689	0.5568318
IB_Control-CY_Control	-0.053666603	-0.147168948	0.0398357428	0.7421079
IB_Treatment-CY_Control	-0.124284544	-0.217786889	-0.0307821987	0.0013170
IR_Control-CY_Control	0.148992266	0.055489921	0.2424946116	0.0000392
IR_Treatment-CY_Control	0.064821820	-0.028680525	0.1583241658	0.4683598
ST_Control-CY_Control	0.195845240	0.102342894	0.2893475849	0.0000000
ST_Treatment-CY_Control	0.245051329	0.151548983	0.3385536741	0.0000000
IB_Control-CY_Treatment	-0.114947726	-0.208450071	-0.0214453808	0.0043973
IB_Treatment-CY_Treatment	-0.185565668	-0.279068013	-0.0920633223	0.0000001
IR_Control-CY_Treatment	0.087711143	-0.005791203	0.1812134881	0.0874065
IR_Treatment-CY_Treatment	0.003540697	-0.089961648	0.0970430423	1.0000000
ST_Control-CY_Treatment	0.134564116	0.041061771	0.2280664614	0.0003213
ST_Treatment-CY_Treatment	0.183770205	0.090267860	0.2772725505	0.0000002
IB_Treatment-IB_Control	-0.070617941	-0.164120287	0.0228844038	0.3345757
IR_Control-IB_Control	0.202658869	0.109156524	0.2961612142	0.0000000
IR_Treatment-IB_Control	0.118488423	0.024986078	0.2119907684	0.0028094
ST_Control-IB_Control	0.249511842	0.156009497	0.3430141875	0.0000000
ST_Treatment-IB_Control	0.298717931	0.205215586	0.3922202766	0.0000000
IR_Control-IB_Treatment	0.273276810	0.179774465	0.3667791557	0.0000000
IR_Treatment-IB_Treatment	0.189106365	0.095604019	0.2826087099	0.0000001
ST_Control-IB_Treatment	0.320129784	0.226627438	0.4136321290	0.0000000
ST_Treatment-IB_Treatment	0.369335873	0.275833527	0.4628382181	0.0000000
IR_Treatment-IR_Control	-0.084170446	-0.177672791	0.0093318995	0.1200576
ST_Control-IR_Control	0.046852973	-0.046649372	0.1403553186	0.8733440
ST_Treatment-IR_Control	0.096059062	0.002556717	0.1895614078	0.0385066
ST_Control-IR_Treatment	0.131023419	0.037521074	0.2245257644	0.0005271
ST_Treatment-IR_Treatment	0.180229508	0.086727163	0.2737318536	0.0000003
ST_Treatment-ST_Control	0.049206089	-0.044296256	0.1427084345	0.8333861

Table S5.5. Statistical output of PERMANOVA (adonis2, vegan package) testing the effect of sampling time on the microbiome composition for each host species within treatment groups (10,000 permutations).

```
adonis2(formula = d ~ SamplingTimepoint, data = df, permutations = perm, method =
"bray")
```

	Df	SumOfSqs	F	Pr(>F)
SamplingTimepoint	2	0.197	0.218	0.9988
Residual	105	47.372		

Table S5.6. Statistical output of PERMANOVA (adonis2, vegan package) testing the effect of host genotype on the microbiome composition within each host species (10,000 permutations).

```
adonis2(formula = d ~ Genotype, data = df, permutations = perm, method = "bray")
```

	Df	SumOfSqs	F	Pr(>F)	
Genotype	35	42.356	16.714	9.999e-05	***
Residual	72	5.213			

Table S6.1. 16S rRNA gene amplification using primers 27F and 519R.

A) Preparation of master mix for amplification of 27F and 519R region of the 16S rRNA gene

Component	Volume (µl)	Final concentration
10 x Immolase Buffer	2.5	1x
10 mM dNTP	0.5	200 nM
50 mM $MgCl_2$	1.25	2.5 mM
ILM_27F Uv3 (forward) (5µM)	2.5	500 nM
ILM_519R (reverse) (5µM)	2.5	500 nM
Immolase DNA Polymerase (5U/µl)	0.2	1 Unite
H_2O	14.55	-
Template	1	-
Total Volume	25	-

B) Thermocycler conditions for the amplification of 27F and 519R region of the 16S rRNA gene)

	Temperature (°C)	Time (mm:ss)
Activation	95	10:00
Amplification (35 cycles)	94	00:30
	55	00:10
	72	00:45
Final Extension	72	10:00

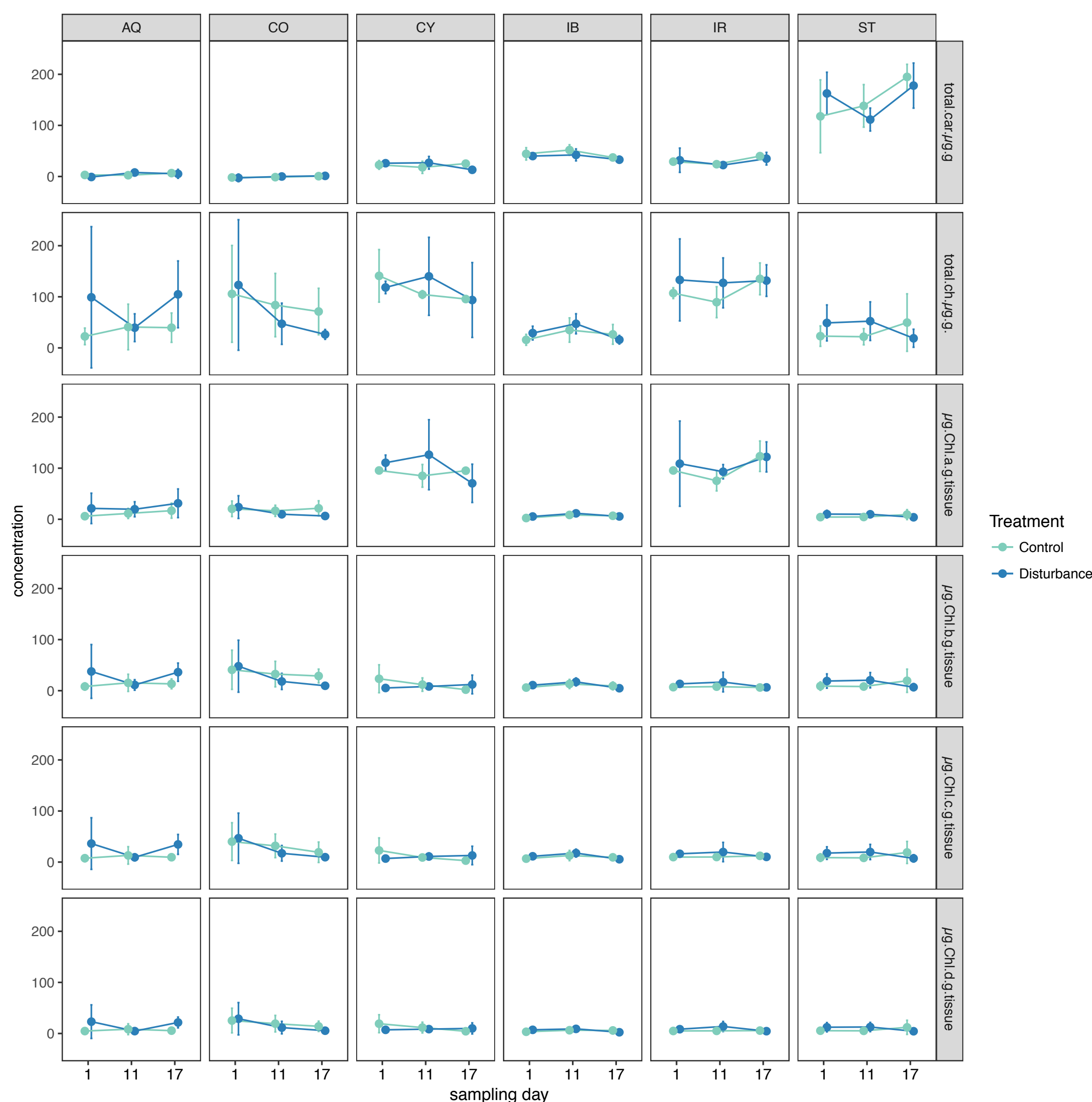


Figure S5.1. Average photopigment concentration (in µg per g sponge tissue) of control and disturbance samples throughout the experiment. Graph displays the total Carotenoids, total Chlorophyll, Chlorophyll a, b, c and d (from top to bottom) concentration for *Amphimedon queenslandica* (AQ), *Coscinoderma matthewsi* (CO), *Cymbastella coralliophila* (CY), *Ianthella basta* (IB), *Ircinia ramosa* (IR) and *Stylissa flabelliformis* (ST) (from left to right). Error bars represent standard error.

Equation S5.1. Equations for photopigment concentration. Chlorophyll a, b, c and d, and total chlorophyll and total carotenoid concentrations (in µg/mL) were calculated using the following equations (Lichtenthaler 1987, Ritchie 2008):

$$Chl\ a = \frac{[(-0.9394 \text{ x E632}) + (-4.2774 \text{ x E649}) + (13.3914 \text{ x E665})]}{0.794}$$

$$Chl\ b = \frac{[(-4.0937\ x\ E632) + (25.6865\ x\ E649) + (-7.3430\ x\ E665)]}{0.794}$$

$$Chl\ c = \frac{[(28.5073\ x\ E632) + (-9.9940\ x\ E649) + (-1.9749\ x\ E665)]}{0.794}$$

$$Chl\ d = \frac{[(-0.2007\ x\ E632) + (0.0848\ x\ E649) + (-0.1909\ x\ E665) + (12.1302\ x\ E696)]}{0.794}$$

$$Total\ Chl = \frac{[(24.1209\ x\ E632) + (11.2884\ x\ E649) + (3.7620\ x\ E665) + (5.8338 x E696)]}{0.794}$$

$$Total\ carotenoids = \frac{[((1000\ x\ E470)\ /\ 0.794) - (2.13\ x\ Chl\ a) - (97.64\ x\ Chl\ b)]}{209}$$

Appendix C – Supplementary Material for Chapter 6

Table S6.2. Statistical output of the TukeyHSD post hoc test (95% confidence interval) used to compare a) protein concentration, b) photochemical efficiency and c) Symbiodiniaceae density between treatment groups (control, acute stress and cumulative stress).

a) Protein concentration

	diff	lwr	upr	p adj
cumulative stress-control	-0.62728959	-1.1800774	-0.07450175	0.0220395
single stress-control	0.08027902	-0.4885350	0.64909302	0.9397306
single stress-cumulative stress	0.70756861	0.1547808	1.26035646	0.0083073

b) Photochemical efficiency

	diff	lwr	upr	p adj
cumulative stress-control	1.0403706	0.5161774	1.5645637	0.0000231
single stress-control	0.3404764	-0.1989139	0.8798667	0.2942703
single stress-cumulative stress	-0.6998942	-1.2240874	-0.1757010	0.0055985

c) Symbiodiniaceae density

	diff	lwr	upr	p adj
cumulative stress-control	-0.6436856	-1.1836395	-0.1037318	0.0151617
single stress-control	0.2429778	-0.3126302	0.7985857	0.5530122
single stress-cumulative stress	0.8866634	0.3467095	1.4266173	0.0005010

Table S6.3. Statistical output of PERMANOVA (adonis2, vegan package) testing the effect of treatment, sampling time point and tank on the microbiome composition within each host genotype (10,000 permutations).

```
Blocks:  with(df, Genotype)
Permutation: free
Number of permutations: 10000
adonis2(formula = d ~ Treatment + SamplingTimepoint + Tank, data = df, permutations
= perm, method = "bray")
```

	Df	SumOfSqs	R2	F	Pr(>F)
Treatment	2	0.5223	0.01993	1.0045	0.2448
SamplingTimepoint	3	0.8291	0.03164	1.0631	0.1496
Tank	6	3.0189	0.11519	1.9354	0.6141
Residual	84	21.8380	0.83325		
Total	95	26.2083	1.00000		

Table S6.4. Statistical output of permutational ANOVA for db-RDA (anova.cca, vegan package) evaluating the contribution to the percent explained observed community variation in the *A. tenuis* microbiome.

	Df	SumOfSqs	F	Pr(>F)	
Genotype	8	6.9107	3.7584	0.000999	***
Treatment	2	0.5902	1.2840	0.142857	
SymbiontDensity	1	0.3569	1.5526	0.072927	.
Protein	1	0.1775	0.7725	0.716284	
Chla	1	0.1945	0.8463	0.612388	
Delta F/Fm’	1	0.2474	1.0766	0.335664	
Residual	81	18.6170			

Table S6.5. Statistical output of PERMANOVA (adonis2, vegan package) testing the effect of treatment, sampling time point and tank on the *Endozoicomonas* community composition within each host genotype (10,000 permutations).

```
Blocks:  with(df, Genotype)
Permutation: free
Number of permutations: 10000
adonis2(formula = d ~ Treatment + SamplingTimepoint + Tank, data = df, permutations
= perm, method = "bray")
```

	Df	SumOfSqs	R2	F	Pr(>F)
Treatment	2	0.2114	0.01253	0.6081	0.7279
SamplingTimepoint	3	0.3192	0.01891	0.6120	0.7725
Tank	6	1.7420	0.10322	1.6699	0.7206
Residual	84	14.6045	0.86534		
Total	95	16.8772	1.00000		

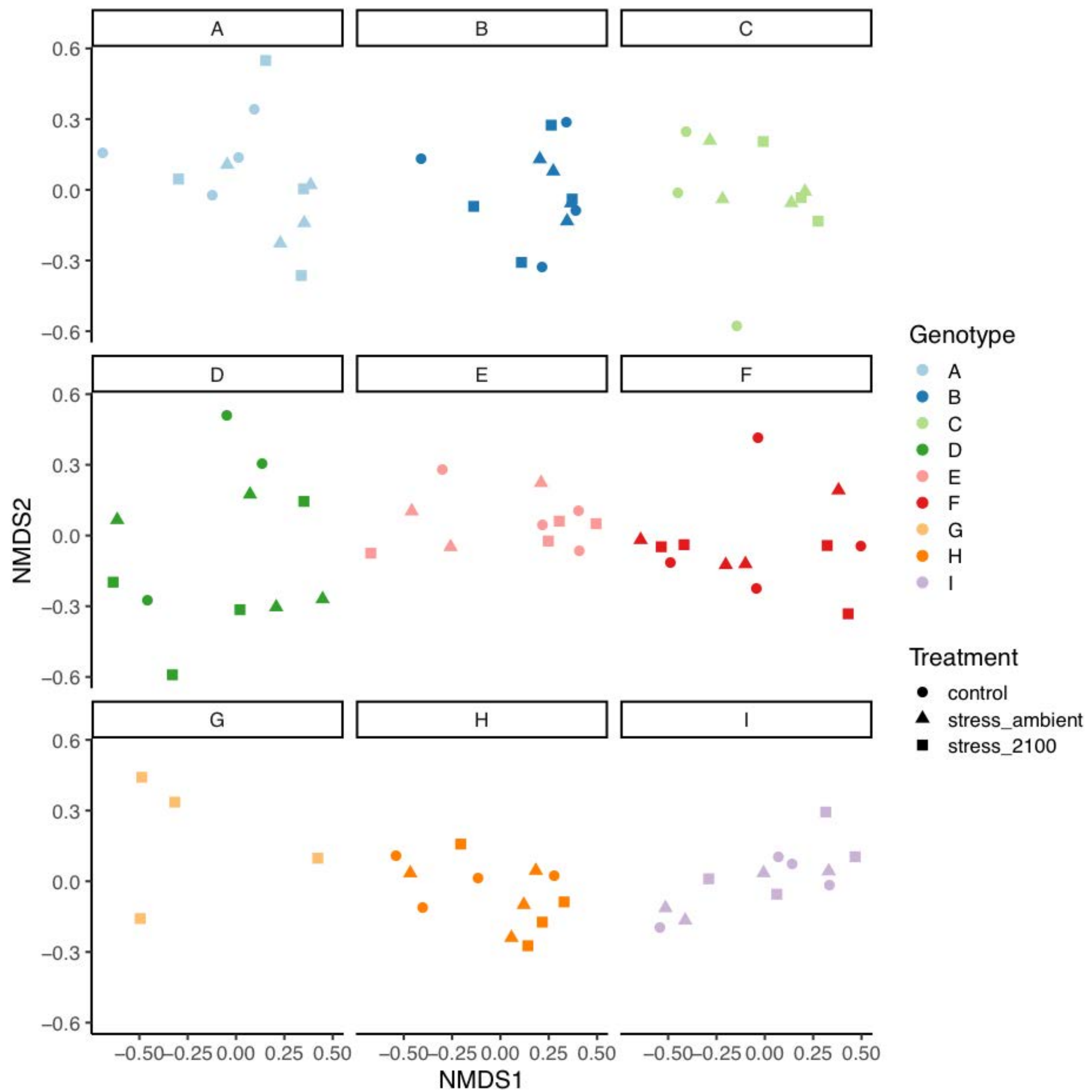


Figure S6.1. Compositional stability of *A. tenuis* microbiome. Non-metric multidimensional scaling (NMDS) plot based on unweighted unifrac distances (only considering the presence/absence of amplicon sequence variants).

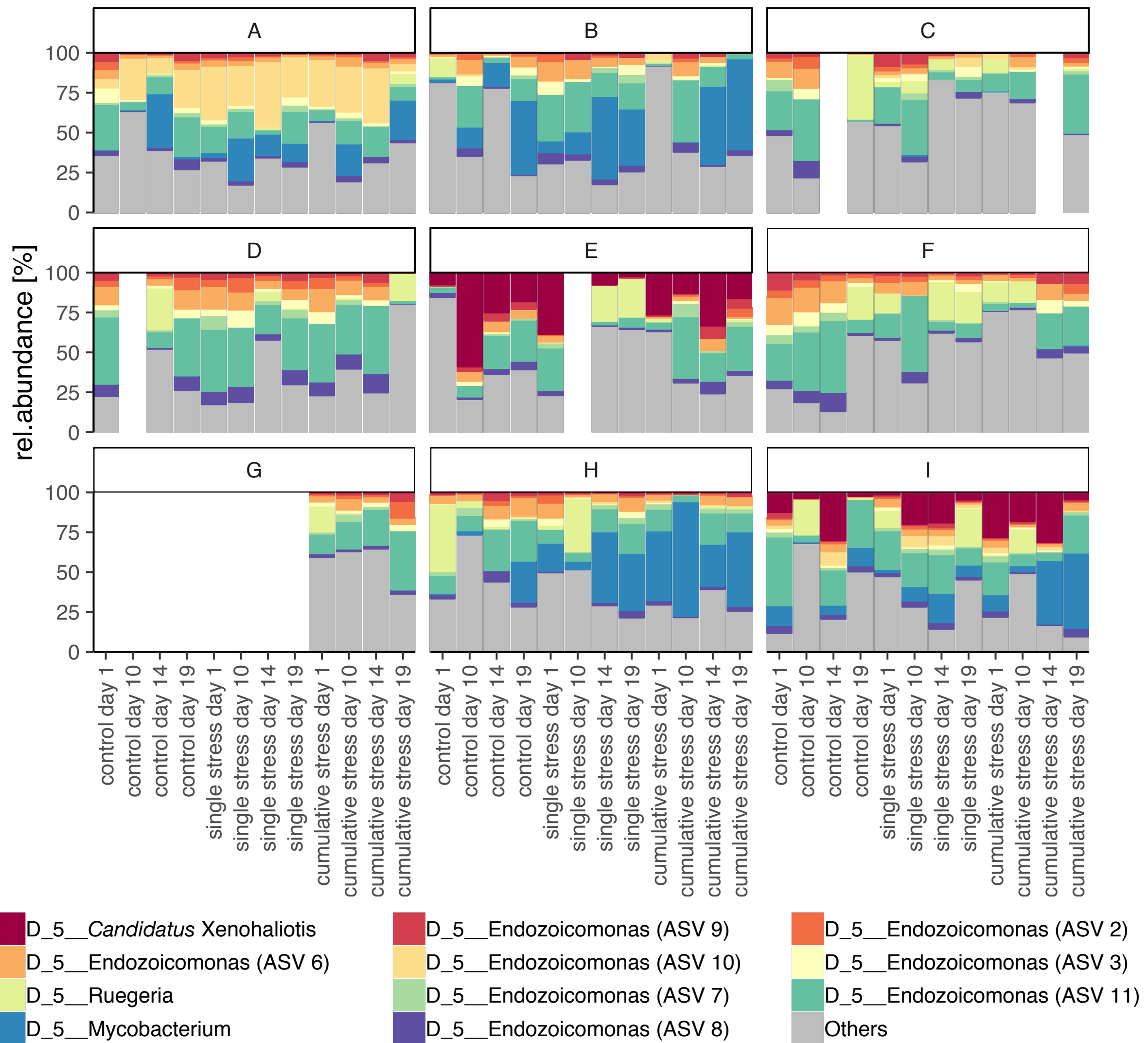


Figure S6.2. Microbiome composition of *A. tenuis*. The relative abundance distribution of the 11 most abundant amplicon sequence variants (ASVs) associated with individual coral nubbins of each host genotype (A-I) under control, single stress and cumulative stress conditions over time (day 1, 10, 14 and 19).

Equation S6.1. Equation for a) Maximum quantum yield and b) Chlorophyll a concentration:

a) Maximum quantum yield (Fv/Fm) was calculated by measuring the minimum (Fo) and maximum (Fm) fluorescence of the Symbiodiniaceae within the coral host tissue.

$$F_v/F_m = (F_m - F_o)/F_m$$

b) Chlorophyll *a* concentrations were calculated using the following equation:

$$Chlorophyll\ a\ (\mu g\ ml^{-1}) = 11.47 \times A_{663} - 0.64 \times A_{630}$$

Appendix D – Supplementary Material for Chapter 7

Table S7.1. Binning results prior to de-replication

habitat	nr of samples	bins total	bins with quality ≥ 50	Completeness (in %)	Contamination (in %)	Total recovery (in %)
sponge	6	271	151	84.01 (± 11.81)	1.97 (± 1.98)	63.5
macroalgae	6	215	52	79.65 (± 14.58)	2.04 (± 2.19)	27.7
seawater	18	1908	765	76.47 (± 12.35)	1.47 (± 1.44)	35.2

Table S7.2. Taxonomic affiliations of de-replicated MAGs (based on 95% Average Nucleotide Identity) using GTDB taxonomy.

a) Sponge $MAGs_{95\%ANI}$

sponge

sponge

Bin_ID	domain	phylum	class	order	family	genus	species
CO36406bin_13	d__Archaea	p__Crenarchaeota	c__Nitrososphaeria	o__Nitrososphaerales	f__Nitrosopumilaceae	g__	
CO36404bin_3	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__Caldilineales	f__Caldilineaceae	g__bin5	
CO36405bin_14	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__Caldilineales	f__Caldilineaceae	g__bin5	
CO36388bin_16	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__SBR1031	f__A4b	g__UBA6055	
CO36386bin_31	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__SBR1031	f__A4b	g__UBA6055	
CO36386bin_32	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__SBR1031	f__A4b	g__UBA6055	
CO36388bin_17	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__SBR1031	f__A4b	g__UBA6055	
CO36386bin_4	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__SBR1031	f__A4b	g__UBA6055	
CO36387bin_22	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__SBR1031	f__	g__	
CO36386bin_13	d__Bacteria	p__Chloroflexota	c__UBA2235	o__UBA11872	f__UBA11872	g__	
CO36404bin_8	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__bin76	f__	g__	
CO36387bin_10	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__bin76	f__	g__	
CO36386bin_14	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__Microtrichales	f__TK06	g__	
CO36406bin_26	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	
CO36386bin_37	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	
CO36386bin_29	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	
CO36386bin_10	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__Pirellulaceae	g__Mariniblastus	
CO36387bin_4	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__Pirellulaceae	g__Rubripirellula	
CO36405bin_2	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__UBA1268	g__UBA1268	
CO36405bin_5	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__UBA1268	g__	
CO36404bin_19	d__Bacteria	p__Acidobacteriota	c__Luteitaleia	o__Luteitaleales	f__UBA8438	g__	
CO36386bin_12	d__Bacteria	p__Acidobacteriota	c__Luteitaleia	o__Luteitaleales	f__UBA8438	g__	
CO36388bin_9	d__Bacteria	p__Acidobacteriota	c__Thermoanaerobaculia	o__	f__	g__	
CO36388bin_1	d__Bacteria	p__Acidobacteriota	c__Thermoanaerobaculia	o__	f__	g__	
CO36406bin_18	d__Bacteria	p__UBP10	c__GR-WP33-30	o__bin18	f__bin18	g__bin18	
CO36406bin_19	d__Bacteria	p__UBP10	c__GR-WP33-30	o__bin18	f__bin18	g__	
CO36405bin_1	d__Bacteria	p__Nitrospirota	c__Nitrospiria	o__Nitrospirales	f__UBA8639	g__bin75	
CO36386bin_35	d__Bacteria	p__Nitrospinota	c__UBA8248	o__UBA8248	f__UBA8248	g__bin107	
CO36404bin_6	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__UBA828	f__	g__	
CO36404bin_4	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhodobacterales	f__Rhodobacteraceae	g__bin36	
CO36386bin_9	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__bin65	f__bin65	g__bin65	
CO36386bin_3	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__UBA10353	f__LS-SOB	g__	
CO36405bin_22	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__UBA6522	f__UBA6522	g__	
CO36388bin_2	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__UBA11654	f__UBA11654	g__	
CO36386bin_20	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__	f__	g__	
CO36386bin_8	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Coxiellales	f__Coxiellaceae	g__	
CO36388bin_10	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__HTCC2089	g__bin55	
CO36404bin_7	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__HTCC2089	g__bin55	

b) Macroalgae $MAGs_{95\%ANI}$

macroalgae

Bin_ID	domain	phylum	class	order	family	genus	species
SS36439bin_8	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae	g__Bacillus	s__Bacillus licheniformis
SS36439bin_9	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae_A	g__Bacillus_AC	s__Bacillus_AC circulans_A
SS36440bin_1	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae_H	g__Bacillus_C	s__Bacillus_C aryabhattai_A
SS36439bin_7	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae	g__Bacillus	s__
SS36441bin_5	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae	g__Bacillus_AY	s__Bacillus_AY weihaiensis
SS36439bin_4	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae_H	g__Bacillus_C	s__Bacillus_C megaterium
SS36427bin_1	d__Bacteria	p__Chloroflexota	c__Anaerolineae	o__Promineofilales	f__	g__	s__
SS36439bin_2	d__Bacteria	p__Actinobacteriota	c__Actinobacteria	o__Actinomycetales	f__Demequinaceae	g__	s__
SS36427bin_3	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae_A	g__Bacillus_AC	s__
SS36439bin_11	d__Bacteria	p__Firmicutes	c__Bacilli	o__Bacillales	f__Bacillaceae_A	g__Bacillus_AK	s__
SS36440bin_2	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Cyanobacteriales	f__Coleofasciculaceae	g__Moorea	s__
SS36425bin_4	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__Aquimarina	s__
SS36427bin_21	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__Aquimarina	s__
SS36425bin_2	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Cytophagales	f__Amoebophilaceae	g__	s__
SS36425bin_5	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Cytophagales	f__	g__	s__
SS36427bin_12	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Verrucomicrobiales	f__Akkermansiaceae	g__	s__
SS36427bin_6	d__Bacteria	p__Spirochaetota	c__Spirochaetia	o__Spirochaetales	f__Alkalispirochaetaceae	g__	s__
SS36427bin_2	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhizobiales	f__	g__	s__
SS36439bin_6	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Sphingomonadales	f__Sphingomonadaceae	g__Altererythrobacter	s__
SS36439bin_13	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__UBA10353	f__	g__	s__

macr

c) Seawater MAGs$_{95\%ANI}$

seawater

PL34863bin_7	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIB	g__UBA9562	s__GCA_002696615.1
PL34863bin_27	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIB	g__UBA252	s__GCA_001628435.1
PL36323bin_28	d__Archaea	p__Asgardarchaeota	c__Heimdallarchaeia	o__LC-2	f__LC-2	g__GCA-2728275	s__GCA_002728275.1
PL34863bin_5	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIB	g__UBA557	s__
PL34864bin_10	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIB	g__UBA11751	s__
PL36325bin_14	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIA	g__UBA562	s__
PL36329bin_3	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIA	g__UBA253	s__
PL36325bin_29	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIA	g__UBA120	s__
PL36311bin_9	d__Archaea	p__Thermoplasmatota	c__MGII	o__MGII	f__MGIIA	g__UBA120	s__
PL34865bin_12	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__Microtrichales	f__UBA11606	g__MedAcidi-G2A	s__MedAcidi-G2A sp3
PL36330bin_20	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Halieaceae	g__Luminiphilus	s__GCA_002703585.1
PL36330bin_32	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Litoricolaceae	g__Litoricola	s__GCF_000227525.1
PL36311bin_20	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhodobacterales	f__Rhodobacteraceae	g__TMED111	s__TMED111 sp1
PL36325bin_24	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhodobacterales	f__Rhodobacteraceae	g__TMED111	s__
PL36325bin_8	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__Microtrichales	f__UBA11606	g__UBA11606	s__GCA_002690205.1
PL36324bin_7	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhodobacterales	f__Rhodobacteraceae	g__HIMB11	s__GCA_001510135.1
PL36313bin_3	d__Bacteria	p__SAR324	c__SAR324	o__SAR324	f__NAC60-12	g__UBA1014	s__UBA1014 sp2
PL36323bin_1	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__UBA8309	s__UBA8309 sp3
PL34863bin_47	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Nisaeales	f__Nisaeaceae	g__GCA-002701455	s__GCA_002690995.1
PL36312bin_6	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__Microtrichales	f__Ilumatobacteraceae	g__Casp-actino5	s__
PL34863bin_39	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	s__
PL36324bin_3	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Cryomorphaceae	g__UBA10364	s__
PL36326bin_6	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Cryomorphaceae	g__UBA10364	s__
PL36329bin_53	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MED-G14	s__
PL36327bin_25	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36330bin_9	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36330bin_11	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36329bin_6	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36324bin_14	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36329bin_16	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36329bin_2	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36323bin_8	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MS024-2A	s__
PL36326bin_18	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__BACL21	s__
PL36327bin_9	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__UBA3478	s__
PL36313bin_11	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__UA16	g__UBA8752	s__
PL36328bin_37	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__UA16	g__UBA11663	s__
PL36329bin_36	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__UA16	g__UBA11663	s__
PL36312bin_4	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Crocinitomicaceae	g__UBA952	s__
PL36313bin_23	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__UBA10066	g__	s__
PL34864bin_44	d__Bacteria	p__Marinisomatota	c__Marinisomatia	o__Marinisomatales	f__Marinisomataceae	g__Marinisoma	s__
PL36330bin_18	d__Bacteria	p__Planctomycetota	c__UBA1135	o__UBA1135	f__GCA-002686595	g__GCA-2686945	s__
PL36329bin_1	d__Bacteria	p__Planctomycetota	c__UBA8108	o__UBA1146	f__UBA1146	g__UBA12191	s__
PL36328bin_29	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__Pirellulaceae	g__Rubripirellula	s__
PL36313bin_20	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Opitutales	f__Puniceicoccaceae	g__GCA-2690565	s__
PL34863bin_23	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Pedosphaerales	f__UBA1096	g__UBA1096	s__
PL34863bin_20	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Pedosphaerales	f__UBA1100	g__UBA1100	s__
PL34863bin_31	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Pedosphaerales	f__	g__	s__
PL34863bin_32	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Verrucomicrobiales	f__DEV007	g__EC70	s__
PL36313bin_19	d__Bacteria	p__SAR324	c__SAR324	o__SAR324	f__NAC60-12	g__JCVI-SCAAA005	s__
PL36325bin_39	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Pelagibacterales	f__Pelagibacteraceae	g__Pelagibacter	s__
PL36310bin_3	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__UBA1172	g__	s__
PL36330bin_25	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__UBA8309	s__
PL36329bin_7	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__	s__
PL36310bin_7	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__	s__
PL36329bin_29	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__	s__
PL36308bin_32	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__	s__
PL36326bin_7	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__	s__
PL36308bin_10	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Puniceispirillales	f__Puniceispirillaceae	g__	s__
PL36324bin_21	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhodobacterales	f__Rhodobacteraceae	g__HIMB11	s__
PL34864bin_26	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__SAR86	f__D2472	g__	s__
PL36313bin_60	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__SAR86	f__SAR86	g__GCA-2707915	s__
PL34864bin_11	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__UBA10353	f__LS-SOB	g__UBA5682	s__
PL36328bin_12	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Xanthomonadales	f__	g__	s__
PL36313bin_15	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Pseudohongiellaceae	g__UBA9145	s__
PL36323bin_13	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Halieaceae	g__Luminiphilus	s__
PL36313bin_41	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Halieaceae	g__Luminiphilus	s__
PL34863bin_51	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Halieaceae	g__Luminiphilus	s__

Table S7.3. Statistical output of differential abundance analysis (DESeq) highlighting significantly enriched microbial phyla (class for Proteobacteria) in a) sponge, b) macroalgae, and c) seawater microbiomes between winter and summer.

a) Sponge microbiome

phylum.class	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
p__Chloroflexota	16850339	-3.993485	0.844069	-4.73123	2.23E-06	2.45E-05

b) macroalgae microbiome

phylum.class	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
c__Alphaproteobacteria	276990.52	-2.311277	0.6133642	-3.768197	1.64E-04	2.47E-04
c__Gammaproteobacteria	43427.11	19.48362	1.0783512	18.067973	5.70E-73	2.56E-72
p__Actinobacteriota	47004.16	1.526713	0.6728611	2.268986	2.33E-02	2.33E-02
p__Bacteroidota	488327.3	-9.193737	2.2798199	-4.032659	5.51E-05	9.93E-05
p__Chloroflexota	81121.98	-7.093359	2.6293516	-2.69776	6.98E-03	8.98E-03
p__Cyanobacteriota	466645.65	22.769968	1.2215184	18.640709	1.50E-77	1.35E-76
p__Firmicutes	12566031.97	6.072401	2.4164859	2.512906	1.20E-02	1.35E-02
p__Spirochaetota	28686.83	-30	3.9067681	-7.678982	1.60E-14	3.61E-14
p__Verrucomicrobiota	64297.23	-18.980284	1.3903511	-13.651432	1.98E-42	5.94E-42

c) seawater

phylum.class	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
c__Alphaproteobacteria	3235902	-0.3797838	0.1126066	-3.372661	7.44E-04	1.17E-03
p__Bacteroidota	4718048.6	1.0013896	0.1693244	5.914031	3.34E-09	1.16E-08
p__Cyanobacteriota	140817.2	-1.1383357	0.2267763	-5.019642	5.18E-07	1.14E-06
p__Marinisomatota	303744.4	1.1451393	0.2831123	4.044823	5.24E-05	9.60E-05
p__Planctomycetota	347855.5	3.1608062	0.1561974	20.235969	4.72E-91	5.19E-90
p__SAR324	180286.2	0.7702925	0.1085726	7.094722	1.30E-12	7.13E-12
p__Thermoplasmatota	674037	-0.4235043	0.151861	-2.788762	5.29E-03	7.28E-03
p__Verrucomicrobiota	325162.6	-0.6196964	0.1054822	-5.874888	4.23E-09	1.16E-08

Table S7.4. Planktonic Bacteriodota MAGs$_{95\%ANI}$ and the presence of GH families (CAZy database).

Bin_ID	GH16	GH37	GH33	GH30_1	GH13	GH17	GH29	GH53	GH5	GH2	GH95	GH18	GH1	GH30	GH65	GH97	GH113	GH63	GH73	GH13_36	GH43_18	GH5_13
PL36312bin_4	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
PL36313bin_11	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0
PL36313bin_23	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
PL36323bin_8	2	0	1	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
PL36324bin_14	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
PL36324bin_3	0	0	1	1	1	2	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
PL36326bin_18	0	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PL36326bin_6	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
PL36327bin_25	2	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
PL36327bin_9	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0
PL36328bin_37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PL36329bin_16	2	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0
PL36329bin_2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PL36329bin_36	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PL36329bin_53	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
PL36329bin_6	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
PL36330bin_11	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
PL36330bin_9	2	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0

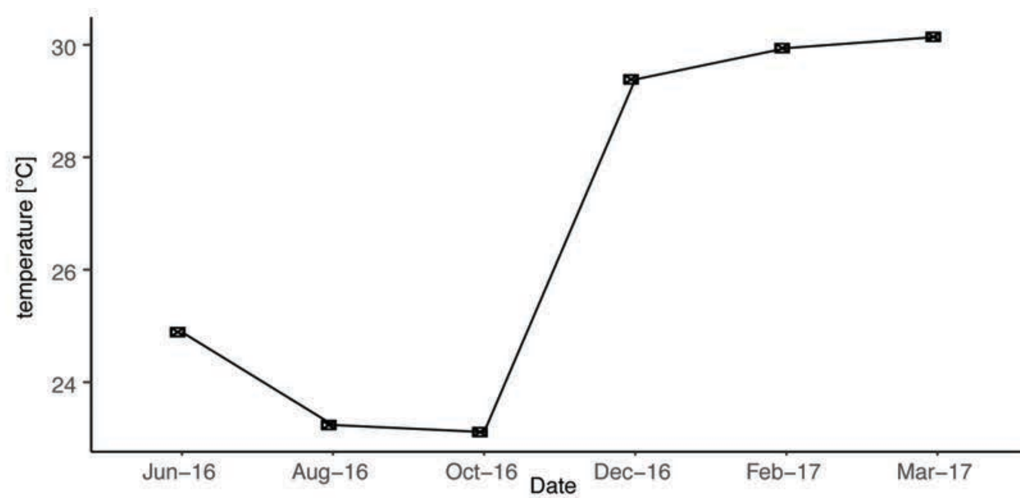
Table S7.5. Planktonic Bacteriodota MAGs$_{95\%ANI}$ and the presence of SusD-like genes (Pfam database).

Bin_ID	PF07980.10	PF12741.6	PF14322.5	PF12771.6
PL36312bin_4	2	0	2	0
PL36313bin_11	2	1	3	2
PL36313bin_23	2	1	2	1
PL36323bin_8	1	2	3	2
PL36324bin_14	4	1	5	2
PL36324bin_3	2	1	3	1
PL36326bin_18	2	1	2	1
PL36326bin_6	4	3	6	3
PL36327bin_25	5	0	6	3
PL36327bin_9	3	1	3	1
PL36328bin_37	2	0	1	0
PL36329bin_16	8	1	7	3
PL36329bin_2	5	1	4	2
PL36329bin_36	2	0	2	0
PL36329bin_53	1	1	2	1
PL36329bin_6	4	2	5	2
PL36330bin_11	2	2	5	3
PL36330bin_9	4	2	4	2

Sponge MAGs	Domain	Phylum	Class	Order	Family	Genus	seawater samples						macroalgae samples					
							Aug-16	Aug-16	Aug-16	Feb-17	Feb-17	Feb-17	Aug-16	Aug-16	Aug-16	Feb-17	Feb-17	Feb-17
CO36386bin_10	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__Pirellulaceae	g__Mariniblastus	1	1	1	0	0	0	1	0	1	0	0	0
CO36386bin_29	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	1	1	1	1	1	1	0	0	1	0	0	0
CO36386bin_37	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	1	1	1	0	0	0	0	0	0	0	0	0
CO36386bin_8	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Coxiellales	f__Coxiellaceae	g__	1	1	1	0	0	0	0	0	0	0	0	0
CO36386bin_9	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__bin65	f__bin65	g__bin65	0	0	0	0	0	0	0	0	0	0	0	0
CO36387bin_4	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__Pirellulaceae	g__Rubripirellula	1	1	1	1	1	1	1	1	1	0	0	0
CO36388bin_9	d__Bacteria	p__Acidobacteriota	c__Thermoanaerobaculia	o__	f__	g__	0	0	0	0	0	0	1	0	1	0	0	0
CO36405bin_2	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__UBA1268	g__UBA1268	1	1	1	1	1	1	0	0	1	0	0	0
CO36406bin_26	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	1	1	1	1	1	1	0	0	0	0	0	0

Seawater MAGs	Domain	Phylum	Class	Order	Family	Genus	sponge samples						macroalgae samples					
							Aug-16	Aug-16	Aug-16	Feb-17	Feb-17	Feb-17	Aug-16	Aug-16	Aug-16	Feb-17	Feb-17	Feb-17
PL34863bin_32	d__Bacteria	p__Verrucomicrobiota	c__Verrucomicrobiae	o__Verrucomicrobiales	f__DEV007	g__EC70	1	1	0	0	0	0	1	1	1	0	0	0
PL34863bin_39	d__Bacteria	p__Cyanobacteriota	c__Cyanobacteriia	o__Synechococcales_A	f__Cyanobiaceae	g__Synechococcus_C	1	1	1	1	1	1	0	0	1	0	0	0
PL34863bin_51	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Halieaceae	g__Luminiphilus	1	0	0	0	0	0	0	0	0	0	0	0
PL36312bin_4	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Crocinitomicaceae	g__UBA952	0	0	0	0	0	0	1	1	1	0	0	0
PL36312bin_6	d__Bacteria	p__Actinobacteriota	c__Acidimicrobiia	o__Microtrichales	f__Ilumatobacteraceae	g__Casp-actino5	1	1	0	0	0	0	0	0	0	0	0	0
PL36313bin_11	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__UA16	g__UBA8752	0	0	0	0	0	0	1	1	1	0	0	0
PL36313bin_15	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Pseudohongiel	g__UBA9145	0	0	0	0	0	0	0	0	0	0	0	0
PL36313bin_19	d__Bacteria	p__SAR324	c__SAR324	o__SAR324	f__NAC60-12	g__JCVI-SCAAA005	1	1	0	0	1	1	0	0	0	0	0	0
PL36323bin_13	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Pseudomonadales	f__Halieaceae	g__Luminiphilus	0	0	0	0	0	0	1	1	1	1	0	0
PL36324bin_7	d__Bacteria	p__Proteobacteria	c__Alphaproteobacteria	o__Rhodobacterales	f__Rhodobacteraceae	g__HIMB11	1	0	0	0	0	0	0	0	0	0	0	0
PL36327bin_9	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__UBA3478	0	0	0	0	0	0	1	1	1	1	0	1
PL36328bin_12	d__Bacteria	p__Proteobacteria	c__Gammaproteobacteria	o__Xanthomonadales	f__	g__	0	0	0	1	1	1	1	1	1	1	1	1
PL36328bin_29	d__Bacteria	p__Planctomycetota	c__Planctomycetes	o__Pirellulales	f__Pirellulaceae	g__Rubripirellula	1	1	0	1	1	1	0	0	1	1	0	0
PL36329bin_53	d__Bacteria	p__Bacteroidota	c__Bacteroidia	o__Flavobacteriales	f__Flavobacteriaceae	g__MED-G14	0	0	0	0	0	0	1	1	1	0	0	0

Table S7.6. Presence of MAGs$_{95\%ANI}$ in seawater, macroalgae and sponge samples. Adapter-trimmed reads from samples collected in August 2016 and February 2017 were mapped (75% minimum alignment and 95% minimum identity) against the de-replicated MAGs$_{95\%ANI}$ with coverM v0.2.0 (https://github.com/wwood/CoverM). Nine sponge MAGs$_{95\%ANI}$ were present in seawater and macroalgae samples, 14 seawater MAGs$_{95\%ANI}$ were present in sponge and macroalgae samples and no macroalgae MAGs$_{95\%ANI}$ were found in seawater and sponge samples.



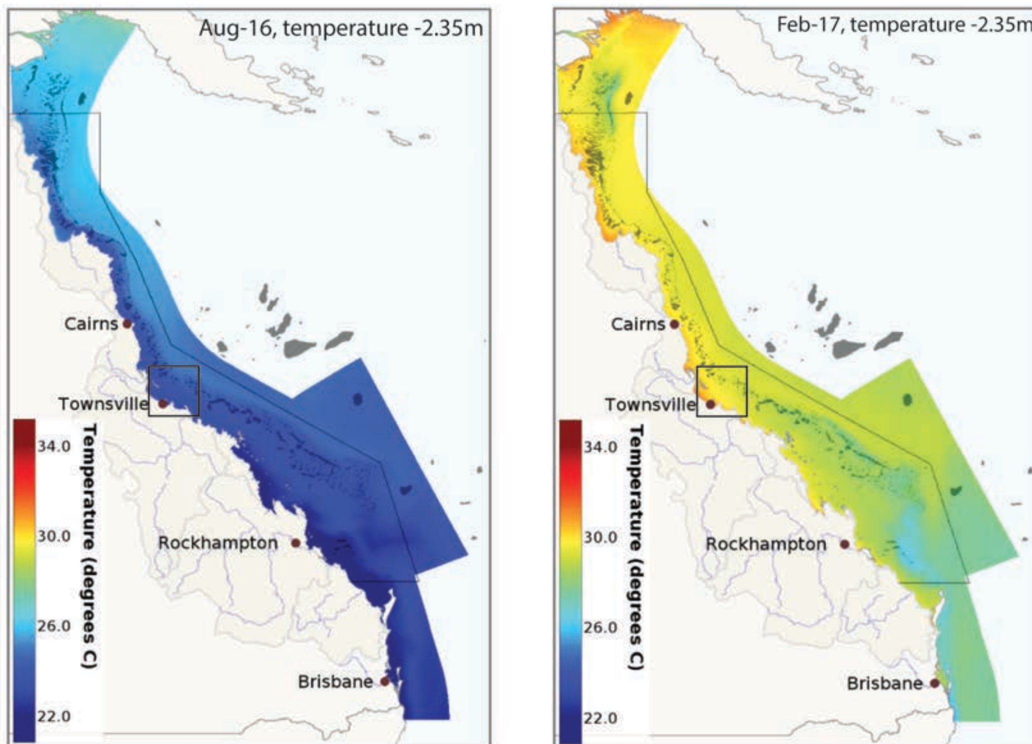


Figure S7.1. Seawater temperature variation between winter (June, August, October) and summer (December, February, March) at the sampling location (Geoffrey Bay, Magnetic Island, Australia). Monthly average seawater temperature in August 2016 and February 2017 along the Great Barrier Reef. Seawater temperature data were retrieved from the eReefs database (**https://aims.ereefs.org.au/)**.

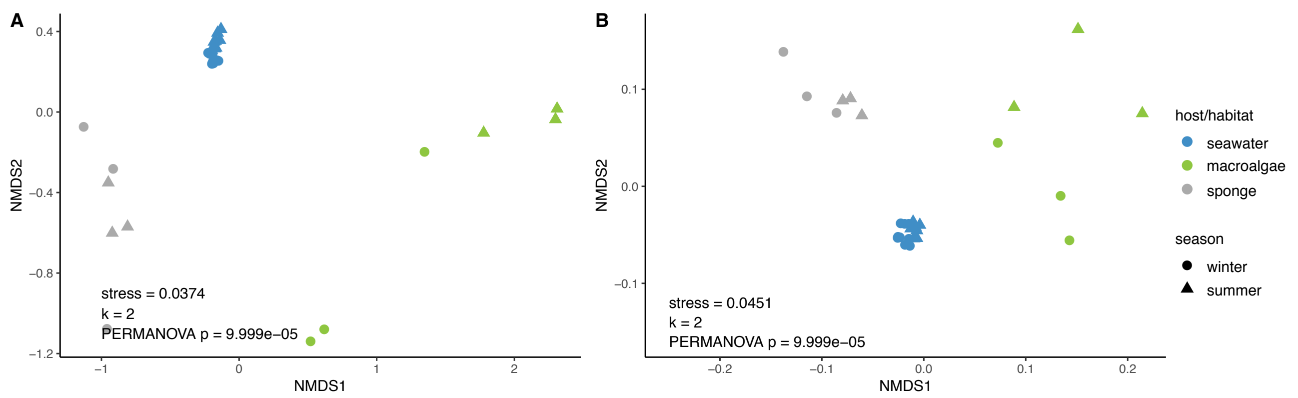


Figure S7.2. Gene-centric approach: taxonomic and functional annotation of metagenome reads. A) Community composition based on adapter trimmed reads was inferred using GraftM v0.12.0 (https://github.com/geronimp/graftM). B) Assembled reads (scaffolds) were used to assess the functional variability of seawater, sponge and macroalgae microbiomes. The functional annotation was performed with enrichM v0.4.7 (https://github.com/geronimp/enrichM) using the KEGG Orthology (KOs). The functional and taxonomic variability between seawater, sponge and macroalgae microbiomes was assessed with PERMANOVAs and Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities in R using the vegan package.

Module	Category	Class
M00787__Bacilysin biosynthesis, prephenate => bacilysin	Biosynthesis of other secondary metabolites	Biosynthesis of other secondary metabolites
M00580__Pentose phosphate pathway, archaea, fructose 6P => ribose 5P	Central carbohydrate metabolism	Carbohydrate metabolism
M00308__Semi−phosphorylative Entner−Doudoroff pathway, gluconate => glycerate−3P		
M00307__Pyruvate oxidation, pyruvate => acetyl−CoA		
M00011__Citrate cycle, second carbon oxidation, 2−oxoglutarate => oxaloacetate		
M00010__Citrate cycle, first carbon oxidation, oxaloacetate => 2−oxoglutarate		
M00009__Citrate cycle (TCA cycle, Krebs cycle)		
M00008__Entner−Doudoroff pathway, glucose−6P => glyceraldehyde−3P + pyruvate		
M00007__Pentose phosphate pathway, non−oxidative phase, fructose 6P => ribose 5P		
M00006__Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P		
M00005__PRPP biosynthesis, ribose 5P => PRPP		
M00004__Pentose phosphate pathway (Pentose phosphate cycle)		
M00003__Gluconeogenesis, oxaloacetate => fructose−6P		
M00002__Glycolysis, core module involving three−carbon compounds		
M00001__Glycolysis (Embden−Meyerhof pathway), glucose => pyruvate		
M00854__Glycogen biosynthesis, glucose−1P => glycogen/starch	Other carbohydrate metabolism	Carbohydrate metabolism
M00741__Propanoyl−CoA metabolism, propanoyl−CoA => succinyl−CoA		
M00632__Galactose degradation, Leloir pathway, galactose => alpha−D−glucose−1P		
M00631__D−Galacturonate degradation (bacteria), D−galacturonate => pyruvate + D−glyceraldehyde 3P		
M00565__Trehalose biosynthesis, D−glucose 1P => trehalose		
M00554__Nucleotide sugar biosynthesis, galactose => UDP−galactose		
M00552__D−galactonate degradation, De Ley−Doudoroff pathway, D−galactonate => glycerate−3P		
M00550__Ascorbate degradation, ascorbate => D−xylulose−5P		
M00549__Nucleotide sugar biosynthesis, glucose => UDP−glucose		
M00373__Ethylmalonyl pathway		
M00061__D−Glucuronate degradation, D−glucuronate => pyruvate + D−glyceraldehyde 3P		
M00013__Malonate semialdehyde pathway, propanoyl−CoA => acetyl−CoA		
M00012__Glyoxylate cycle		
M00417__Cytochrome o ubiquinol oxidase	ATP synthesis	Energy metabolism
M00416__Cytochrome aa3−600 menaquinol oxidase		
M00159__V/A−type ATPase, prokaryotes		
M00157__F−type ATPase, prokaryotes and chloroplasts		
M00156__Cytochrome c oxidase, cbb3−type		
M00155__Cytochrome c oxidase, prokaryotes		
M00151__Cytochrome bc1 complex respiratory unit		
M00150__Fumarate reductase, prokaryotes		
M00149__Succinate dehydrogenase, prokaryotes		
M00145__NAD(P)H:quinone oxidoreductase, chloroplasts and cyanobacteria		
M00144__NADH:quinone oxidoreductase, prokaryotes		
M00579__Phosphate acetyltransferase−acetate kinase pathway, acetyl−CoA => acetate	Carbon fixation	Energy metabolism
M00173__Reductive citrate cycle (Arnon−Buchanan cycle)		
M00172__C4−dicarboxylic acid cycle, NADP − malic enzyme type		
M00169__CAM (Crassulacean acid metabolism), light		
M00168__CAM (Crassulacean acid metabolism), dark		
M00167__Reductive pentose phosphate cycle, glyceraldehyde−3P => ribulose−5P		
M00166__Reductive pentose phosphate cycle, ribulose−5P => glyceraldehyde−3P		
M00165__Reductive pentose phosphate cycle (Calvin cycle)		
M00378__F420 biosynthesis	Methane metabolism	Energy metabolism
M00346__Formaldehyde assimilation, serine pathway		
M00345__Formaldehyde assimilation, ribulose monophosphate pathway		
M00174__Methane oxidation, methanotroph, methane => formaldehyde		
M00531__Assimilatory nitrate reduction, nitrate => ammonia	Nitrogen metabolism	Energy metabolism
M00530__Dissimilatory nitrate reduction, nitrate => ammonia		
M00597__Anoxygenic photosystem II	Photosynthesis	Energy metabolism
M00163__Photosystem I		
M00161__Photosystem II		
M00596__Dissimilatory sulfate reduction, sulfate => H2S	Sulfur metabolism	Energy metabolism
M00595__Thiosulfate oxidation by SOX complex, thiosulfate => sulfate		
M00176__Assimilatory sulfate reduction, sulfate => H2S		
M00817__Lantibiotic transport system	ABC−2 type and other transport systems	Environmental information processing
M00813__Lantibiotic transport system		
M00762__Copper−processing system		
M00747__Bacitracin transport system		
M00634__Oleandomycin transport system		
M00584__Acetoin utilization transport system		
M00320__Lipopolysaccharide export system		
M00315__Uncharacterized ABC transport system		
M00314__Bacitracin transport system		
M00259__Heme transport system		
M00258__Putative ABC transport system		
M00256__Cell division transport system		
M00255__Lipoprotein−releasing system		
M00254__ABC−2 type transport system		
M00253__Sodium transport system		
M00252__Lipooligosaccharide transport system		
M00251__Teichoic acid transport system		
M00250__Lipopolysaccharide transport system		
M00249__Capsular polysaccharide transport system		
M00224__Fluoroquinolone transport system		
M00360__Aminoacyl−tRNA biosynthesis, prokaryotes	Aminoacyl tRNA Metabolism	Environmental information processing
M00359__Aminoacyl−tRNA biosynthesis, eukaryotes		
M00429__Competence−related DNA transformation transporter	Bacterial secretion system	Environmental information processing
M00336__Twin−arginine translocation (Tat) system		
M00335__Sec (secretion) system		
M00333__Type IV secretion system		
M00331__Type II general secretion pathway		
M00330__Adhesin protein transport system		
M00765__Multidrug resistance, efflux pump Bmr	Drug efflux transporter/pump	Environmental information processing
M00738__Bacitracin resistance, BceAB transporter		
M00720__Multidrug resistance, efflux pump VexEF−TolC		
M00717__Multidrug resistance, efflux pump NorA		
M00715__Lincosamide resistance, efflux pump LmrB		
M00712__Multidrug resistance, efflux pump YkkCD		
M00710__Multidrug resistance, efflux pump EbrAB		
M00707__Multidrug resistance, MdlAB/SmdAB transporter		
M00647__Multidrug resistance, efflux pump AcrAB−TolC/SmeDEF		
M00754__Nisin resistance, phage shock protein homolog LiaH	Drug resistance	Environmental information processing
M00743__Aminoglycoside resistance, protease HtpX		
M00742__Aminoglycoside resistance, protease FtsH		
M00728__Cationic antimicrobial peptide (CAMP) resistance, envelope protein folding and degrading factors DegP and DsbA		
M00727__Cationic antimicrobial peptide (CAMP) resistance, N−acetylmuramoyl−L−alanine amidase AmiA and AmiC		
M00628__beta−Lactam resistance, AmpC system		

KEGG Modules

M00792__Manganese/zinc transport system
M00582__Energy−coupling factor transport system
M00581__Biotin transport system
M00319__Manganese/zinc/iron transport system
M00317__Manganese/iron transport system
M00316__Manganese transport system
M00247__Putative ABC transport system
M00246__Nickel transport system
M00245__Cobalt/nickel transport system
M00244__Putative zinc/manganese transport system
M00243__Manganese/iron transport system
M00242__Zinc transport system
M00240__Iron complex transport system

Metallic cation, iron−siderophore and vitamin B12 transport system | Environmental information processing

M00442__Putative hydroxymethylpyrimidine transport system
M00438__Nitrate/nitrite transport system
M00436__Sulfonate transport system
M00435__Taurine transport system
M00300__Putrescine transport system
M00299__Spermidine/putrescine transport system
M00209__Osmoprotectant transport system
M00208__Glycine betaine/proline transport system
M00193__Putative spermidine/putrescine transport system
M00192__Putative thiamine transport system
M00191__Thiamine transport system
M00190__Iron(III) transport system
M00189__Molybdate transport system
M00188__NitT/TauT family transport system
M00186__Tungstate transport system
M00185__Sulfate transport system

Mineral and organic ion transport system | Environmental information processing

M00362__Nucleotide sugar biosynthesis, prokaryotes
M00361__Nucleotide sugar biosynthesis, eukaryotes

Nucleotide metabolism | Environmental information processing

M00566__Dipeptide transport system, Firmicutes
M00440__Nickel transport system
M00439__Oligopeptide transport system
M00349__Microcin C transport system
M00239__Peptides/nickel transport system

Peptide and nickel transport system | Environmental information processing

M00589__Putative lysine transport system
M00586__Putative amino−acid transport system
M00585__L−Cystine transport system
M00323__Urea transport system
M00238__D−Methionine transport system
M00237__Branched−chain amino acid transport system
M00236__Putative polar amino acid transport system
M00234__Cystine transport system
M00233__Glutamate transport system
M00232__General L−amino acid transport system
M00228__Putative glutamine transport system
M00223__Phosphonate transport system
M00222__Phosphate transport system

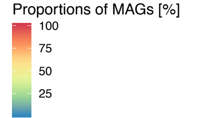
Phosphate and amino acid transport system | Environmental information processing

M00764__PTS system, fructoselysine/glucoselysine−specific II component
M00304__PTS system, fructose−specific II component
M00283__PTS system, ascorbate−specific II component
M00279__PTS system, galactitol−specific II component
M00276__PTS system, mannose−specific II component
M00275__PTS system, cellobiose−specific II component
M00274__PTS system, mannitol−specific II component
M00273__PTS system, fructose−specific II component

Phosphotransferase system (PTS) | Environmental information processing

M00670__Mce transport system
M00669__gamma−Hexachlorocyclohexane transport system
M00607__Glycerol transport system
M00605__Glucose/mannose transport system
M00603__Putative aldouronate transport system
M00602__Arabinosaccharide transport system
M00601__Putative chitobiose transport system
M00600__alpha−1,4−Digalacturonate transport system
M00599__Inositol−phosphate transport system
M00491__arabinogalactan oligomer/maltooligosaccharide transport system
M00221__Putative simple sugar transport system
M00220__Rhamnose transport system
M00218__Fructose transport system
M00216__Multiple sugar transport system
M00215__D−Xylose transport system
M00212__Ribose transport system
M00211__Putative ABC transport system
M00210__Phospholipid transport system
M00207__Putative multiple sugar transport system
M00206__Cellobiose transport system
M00205__N−Acetylglucosamine transport system
M00204__Trehalose/maltose transport system
M00201__alpha−Glucoside transport system
M00200__Putative sorbitol/mannitol transport system
M00199__L−Arabinose/lactose transport system
M00198__Putative sn−glycerol−phosphate transport system
M00197__Putative fructooligosaccharide transport system
M00196__Raffinose/stachyose/melibiose transport system

Saccharide, polyol, and lipid transport system | Environmental information processing



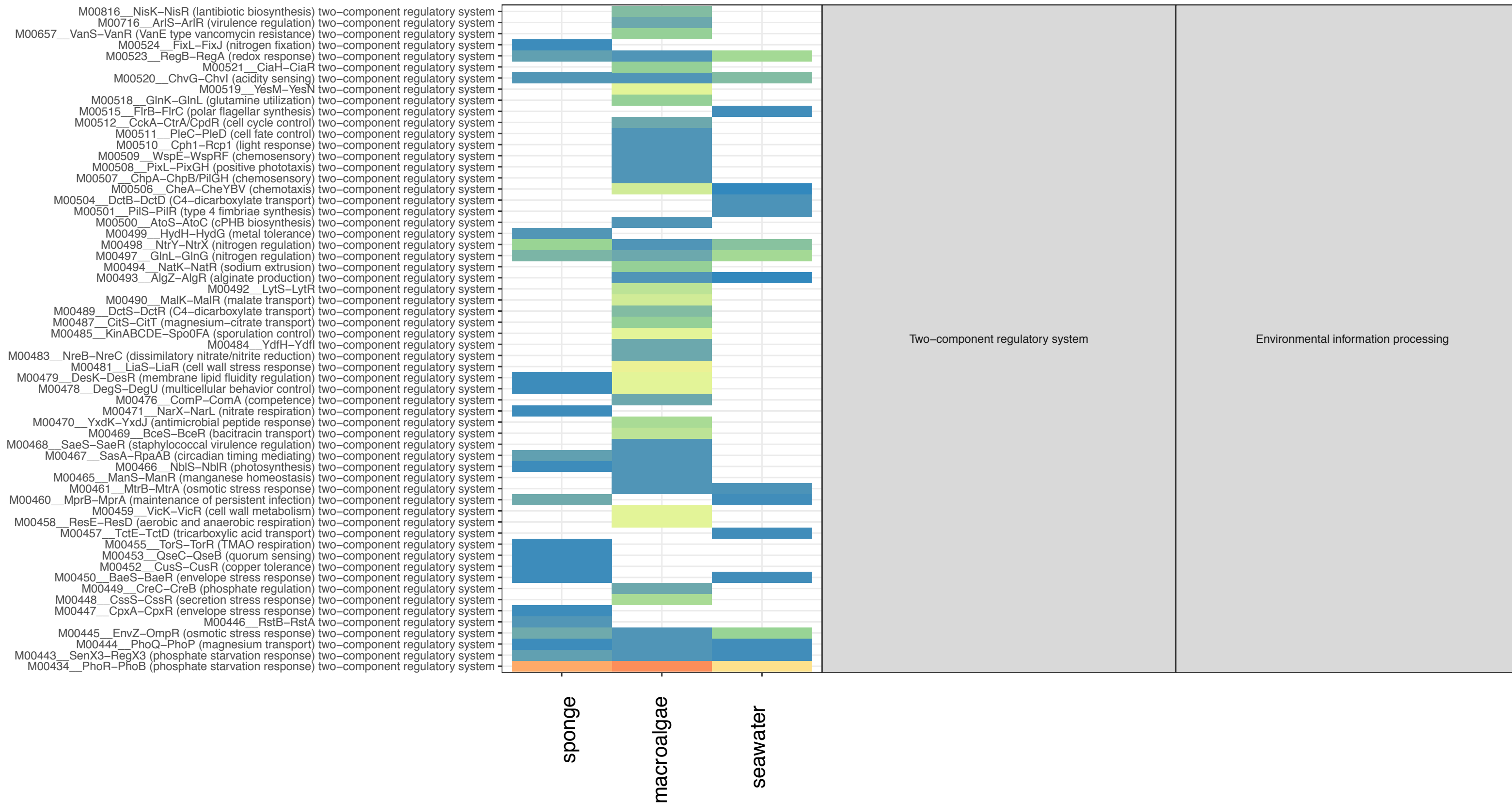


Figure S7.3. Unique and shared KEGG Modules involved in carbohydrate metabolism, energy metabolism, processing of environmental information and production of other secondary metabolites of sponge, algae and seawater microbiomes. Colour represents the relative proportions of MAGs [in %] in a habitat (sponge, macroalgae and seawater) possessing a certain function.

Valine/isoleucine biosynthesis, pyruvate => valine / 2−oxobutanoate => isoleucine
Urea cycle
Tyrosine degradation, tyrosine => homogentisate
Tyrosine biosynthesis, prephanate => pretyrosine => tyrosine
Tryptophan metabolism, tryptophan => kynurenine => 2−aminomuconate
Tryptophan biosynthesis, chorismate => tryptophan
Threonine biosynthesis, aspartate => homoserine => threonine
Shikimate pathway, phosphoenolpyruvate + erythrose−4P => chorismate
Serine biosynthesis, glycerate−3P => serine
Proline biosynthesis, glutamate => proline
Phenylalanine biosynthesis, chorismate => phenylalanine
Ornithine biosynthesis, glutamate => ornithine
Methionine degradation
Methionine biosynthesis, apartate => homoserine => methionine
Lysine degradation, lysine => saccharopine => acetoacetyl−CoA
Lysine biosynthesis, succinyl−DAP pathway, aspartate => lysine
Lysine biosynthesis, DAP dehydrogenase pathway, aspartate => lysine
Lysine biosynthesis, DAP aminotransferase pathway, aspartate => lysine
Leucine degradation, leucine => acetoacetate + acetyl−CoA
Leucine biosynthesis, 2−oxoisovalerate => 2−oxoisocaproate
Isoleucine biosynthesis, threonine => 2−oxobutanoate => isoleucine
Isoleucine biosynthesis, pyruvate => 2−oxobutanoate
Histidine degradation, histidine => N−formiminoglutamate => glutamate
Histidine biosynthesis, PRPP => histidine
Glutathione biosynthesis, glutamate => glutathione
Cysteine biosynthesis, serine => cysteine
Cysteine biosynthesis, homocysteine + serine => cysteine
Betaine biosynthesis, choline => betaine
Arginine biosynthesis, ornithine => arginine
Arginine biosynthesis, glutamate => acetylcitrulline => arginine

Amino acid metabolism

dTDP−L−rhamnose biosynthesis
C5 isoprenoid biosynthesis, non−mevalonate pathway
C5 isoprenoid biosynthesis, mevalonate pathway
C10−C20 isoprenoid biosynthesis, bacteria

Biosynthesis of terpenoids and polyketides

Semi−phosphorylative Entner−Doudoroff pathway, gluconate => glycerate−3P
Pyruvate oxidation, pyruvate => acetyl−CoA
PRPP biosynthesis, ribose 5P => PRPP
Propanoyl−CoA metabolism, propanoyl−CoA => succinyl−CoA
Pentose phosphate pathway, non−oxidative phase, fructose 6P => ribose 5P
Pentose phosphate pathway (Pentose phosphate cycle)
Nucleotide sugar biosynthesis, galactose => UDP−galactose
Malonate semialdehyde pathway, propanoyl−CoA => acetyl−CoA
Glyoxylate cycle
Glycolysis, core module involving three−carbon compounds
Glycolysis (Embden−Meyerhof pathway), glucose => pyruvate
Gluconeogenesis, oxaloacetate => fructose−6P
Galactose degradation, Leloir pathway, galactose => alpha−D−glucose−1P
Ethylmalonyl pathway
Entner−Doudoroff pathway, glucose−6P => glyceraldehyde−3P + pyruvate
D−Glucuronate degradation, D−glucuronate => pyruvate + D−glyceraldehyde 3P
D−Galacturonate degradation (bacteria), D−galacturonate => pyruvate + D−glyceraldehyde 3P
D−galactonate degradation, De Ley−Doudoroff pathway, D−galactonate => glycerate−3P
Citrate cycle, second carbon oxidation, 2−oxoglutarate => oxaloacetate
Citrate cycle, first carbon oxidation, oxaloacetate => 2−oxoglutarate
Citrate cycle (TCA cycle, Krebs cycle)

Carbohydrate metabolism

Thiosulfate oxidation by SOX complex, thiosulfate => sulfate
Succinate dehydrogenase, prokaryotes
Reductive pentose phosphate cycle, ribulose−5P => glyceraldehyde−3P
Reductive pentose phosphate cycle, glyceraldehyde−3P => ribulose−5P
Reductive pentose phosphate cycle (Calvin cycle)
Reductive citrate cycle (Arnon−Buchanan cycle)
Phosphate acetyltransferase−acetate kinase pathway, acetyl−CoA => acetate
NADH:quinone oxidoreductase, prokaryotes
Fumarate reductase, prokaryotes
Formaldehyde assimilation, serine pathway
F420 biosynthesis
F−type ATPase, prokaryotes and chloroplasts
Cytochrome c oxidase, prokaryotes
Cytochrome c oxidase, cbb3−type
Cytochrome bc1 complex respiratory unit
CAM (Crassulacean acid metabolism), light
CAM (Crassulacean acid metabolism), dark
C4−dicarboxylic acid cycle, NADP − malic enzyme type
Anoxygenic photosystem II

Energy metabolism

KEGG Module

Zinc transport system
Twin−arginine translocation (Tat) system
Tungstate transport system
Thiamine transport system
TctE−TctD (tricarboxylic acid transport) two−component regulatory system
Taurine transport system
Spermidine/putrescine transport system
Sec (secretion) system
Rhamnose transport system
RegB−RegA (redox response) two−component regulatory system
Putrescine transport system
Putative zinc/manganese transport system
Putative thiamine transport system
Putative spermidine/putrescine transport system
Putative sorbitol/mannitol transport system
Putative sn−glycerol−phosphate transport system
Putative simple sugar transport system
Putative polar amino acid transport system
Putative multiple sugar transport system
Putative hydroxymethylpyrimidine transport system
Putative aldouronate transport system
Putative ABC transport system
PTS system, mannitol−specific II component
Phosphonate transport system
Phospholipid transport system
Phosphate transport system
PhoR−PhoB (phosphate starvation response) two−component regulatory system
Peptides/nickel transport system
Nucleotide sugar biosynthesis, prokaryotes
NtrY−NtrX (nitrogen regulation) two−component regulatory system
NitT/TauT family transport system
Multidrug resistance, SmdAB/MdlAB transporter
Multidrug resistance, efflux pump VexEF−TolC
Molybdate transport system
Microcin C transport system
Mce transport system
Manganese/zinc/iron transport system
Manganese/iron transport system
Lipoprotein−releasing system
Lipopolysaccharide export system
Iron(III) transport system
Iron complex transport system
Heme transport system
Glycine betaine/proline transport system
Glycerol transport system
Glucose/mannose transport system
GlnL−GlnG (nitrogen regulation) two−component regulatory system
General L−amino acid transport system
gamma−Hexachlorocyclohexane transport system
Fructose transport system
EnvZ−OmpR (osmotic stress response) two−component regulatory system
DctB−DctD (C4−dicarboxylate transport) two−component regulatory system
ChvG−ChvI (acidity sensing) two−component regulatory system
Cell division transport system
Branched−chain amino acid transport system
Biotin transport system
Aminoglycoside resistance, protease FtsH
Aminoacyl−tRNA biosynthesis, prokaryotes
Aminoacyl−tRNA biosynthesis, eukaryotes
alpha−Glucoside transport system
ABC−2 type transport system

Environmental information processing

RNA polymerase, bacteria
RNA degradosome
Ribosome, bacteria
DNA polymerase III complex, bacteria

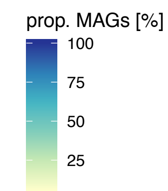
Genetic information processing

Keratan sulfate degradation
KDO2−lipid A biosynthesis, Raetz pathway, LpxL−LpxM type
CMP−KDO biosynthesis
ADP−L−glycero−D−manno−heptose biosynthesis

Glycan metabolism

Phosphatidylethanolamine (PE) biosynthesis, PA => PS => PE
Fatty acid biosynthesis, initiation
Fatty acid biosynthesis, elongation
beta−Oxidation, acyl−CoA synthesis
beta−Oxidation

Lipid metabolism



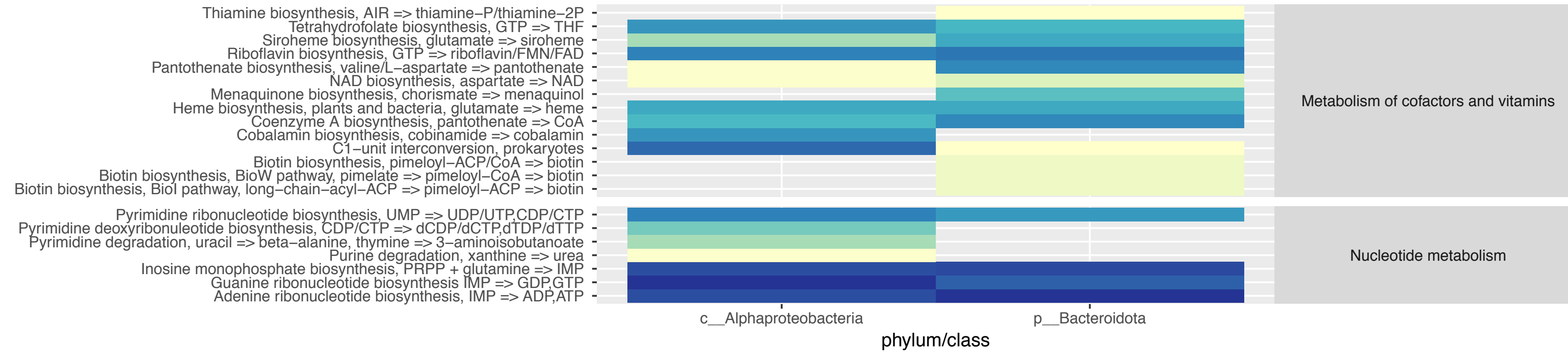
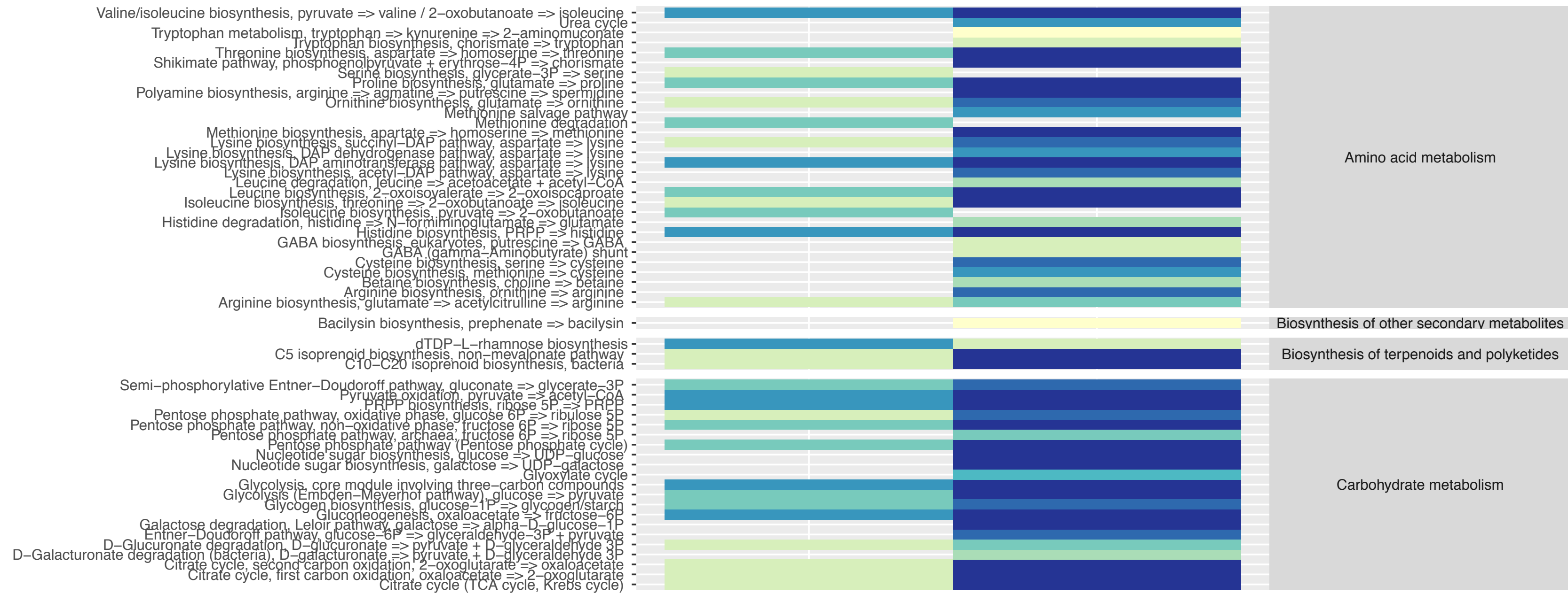


Figure S7.4. KEGG Modules of Alphaproteobacteria and Bacteroidota MAGs$_{95\%ANI}$ derived from seawater. Colour indicates the number of MAGs$_{95\%ANI}$ (in %) that were associated with individual KEGG Modules.



Valine/isoleucine biosynthesis, pyruvate => valine / 2−oxobutanoate => isoleucine
Urea cycle
Tryptophan metabolism, tryptophan => kynurenine => 2−aminomuconate
Tryptophan biosynthesis, chorismate => tryptophan
Threonine biosynthesis, aspartate => homoserine => threonine
Shikimate pathway, phosphoenolpyruvate + erythrose−4P => chorismate
Serine biosynthesis, glycerate−3P => serine
Proline biosynthesis, glutamate => proline
Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine
Ornithine biosynthesis, glutamate => ornithine
Methionine salvage pathway
Methionine degradation
Methionine biosynthesis, apartate => homoserine => methionine
Lysine biosynthesis, succinyl−DAP pathway, aspartate => lysine
Lysine biosynthesis, DAP dehydrogenase pathway, aspartate => lysine
Lysine biosynthesis, DAP aminotransferase pathway, aspartate => lysine
Lysine biosynthesis, acetyl−DAP pathway, aspartate => lysine
Leucine degradation, leucine => acetoacetate + acetyl−CoA
Leucine biosynthesis, 2−oxoisovalerate => 2−oxoisocaproate
Isoleucine biosynthesis, threonine => 2−oxobutanoate => isoleucine
Isoleucine biosynthesis, pyruvate => 2−oxobutanoate
Histidine degradation, histidine => N−formiminoglutamate => glutamate
Histidine biosynthesis, PRPP => histidine
GABA biosynthesis, eukaryotes, putrescine => GABA
GABA (gamma−Aminobutyrate) shunt
Cysteine biosynthesis, serine => cysteine
Cysteine biosynthesis, methionine => cysteine
Betaine biosynthesis, choline => betaine
Arginine biosynthesis, ornithine => arginine
Arginine biosynthesis, glutamate => acetylcitrulline => arginine
Bacilysin biosynthesis, prephenate => bacilysin
dTDP−L−rhamnose biosynthesis
C5 isoprenoid biosynthesis, non−mevalonate pathway
C10−C20 isoprenoid biosynthesis, bacteria
Semi−phosphorylative Entner−Doudoroff pathway, gluconate => glycerate−3P
Pyruvate oxidation, pyruvate => acetyl−CoA
PRPP biosynthesis, ribose 5P => PRPP
Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P
Pentose phosphate pathway, non−oxidative phase, fructose 6P => ribose 5P
Pentose phosphate pathway, archaea, fructose 6P => ribose 5P
Pentose phosphate pathway (Pentose phosphate cycle)
Nucleotide sugar biosynthesis, glucose => UDP−glucose
Nucleotide sugar biosynthesis, galactose => UDP−galactose
Glyoxylate cycle
Glycolysis, core module involving three−carbon compounds
Glycolysis (Embden−Meyerhof pathway), glucose => pyruvate
Glycogen biosynthesis, glucose−1P => glycogen/starch
Gluconeogenesis, oxaloacetate => fructose−6P
Galactose degradation, Leloir pathway, galactose => alpha−D−glucose−1P
Entner−Doudoroff pathway, glucose−6P => glyceraldehyde−3P + pyruvate
D−Glucuronate degradation, D−glucuronate => pyruvate + D−glyceraldehyde 3P
D−Galacturonate degradation (bacteria), D−galacturonate => pyruvate + D−glyceraldehyde 3P
Citrate cycle, second carbon oxidation, 2−oxoglutarate => oxaloacetate
Citrate cycle, first carbon oxidation, oxaloacetate => 2−oxoglutarate
Citrate cycle (TCA cycle, Krebs cycle)
Amino acid metabolism
Biosynthesis of other secondary metabolites
Biosynthesis of terpenoids and polyketides
Carbohydrate metabolism

KEGG Module

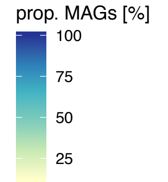
Succinate dehydrogenase, prokaryotes
Reductive pentose phosphate cycle, glyceraldehyde−3P => ribulose−5P
Reductive pentose phosphate cycle (Calvin cycle)
Reductive citrate cycle (Arnon−Buchanan cycle)
Phosphate acetyltransferase−acetate kinase pathway, acetyl−CoA => acetate
NADH:quinone oxidoreductase, prokaryotes
Formaldehyde assimilation, ribulose monophosphate pathway
F−type ATPase, prokaryotes and chloroplasts
Dissimilatory nitrate reduction, nitrate => ammonia
Cytochrome c oxidase, prokaryotes
Cytochrome bc1 complex respiratory unit
Cytochrome aa3−600 menaquinol oxidase
CAM (Crassulacean acid metabolism), light
CAM (Crassulacean acid metabolism), dark
C4−dicarboxylic acid cycle, NADP − malic enzyme type
Assimilatory sulfate reduction, sulfate => H2S

Energy metabolism

Zinc transport system
YxdK−YxdJ (antimicrobial peptide response) two−component regulatory system
YesM−YesN two−component regulatory system
YdfH−YdfI two−component regulatory system
VicK−VicR (cell wall metabolism) two−component regulatory system
VanS−VanR (VanE type vancomycin resistance) two−component regulatory system
Urea transport system
Uncharacterized ABC transport system
Twin−arginine translocation (Tat) system
Teichoic acid transport system
Taurine transport system
Sulfonate transport system
Sulfate transport system
Spermidine/putrescine transport system
Sodium transport system
Sec (secretion) system
SaeS−SaeR (staphylococcal virulence regulation) two−component regulatory system
Ribose transport system
Rhamnose transport system
ResE−ResD (aerobic and anaerobic respiration) two−component regulatory system
Putative spermidine/putrescine transport system
Putative sn−glycerol−phosphate transport system
Putative simple sugar transport system
Putative S−methylcysteine transport system
Putative polar amino acid transport system
Putative multiple sugar transport system
Putative lysine transport system
Putative hydroxymethylpyrimidine transport system
Putative fructooligosaccharide transport system
Putative chitobiose transport system
Putative aldouronate transport system
Putative ABC transport system
PTS system, mannose−specific II component
PTS system, galactitol−specific II component
PTS system, fructoselysine and glucoselysine−specific II component
PTS system, fructose−specific II component
PTS system, cellobiose−specific II component
PTS system, ascorbate−specific II component
Phosphonate transport system
Phosphate transport system
PhoR−PhoB (phosphate starvation response) two−component regulatory system
Peptides/nickel transport system
Osmoprotectant transport system
Oligopeptide transport system
Nucleotide sugar biosynthesis, prokaryotes
NreB−NreC (dissimilatory nitrate/nitrite reduction) two−component regulatory system
NitT/TauT family transport system
Nitrate/nitrite transport system
NisK−NisR (lantibiotic biosynthesis) two−component regulatory system
Nisin resistance, phage shock protein homolog LiaH
Nickel transport system
NatK−NatR (sodium extrusion) two−component regulatory system
Multiple sugar transport system
Multidrug resistance, SmdAB/MdlAB transporter
Multidrug resistance, efflux pump YkkCD
Multidrug resistance, efflux pump NorA
Multidrug resistance, efflux pump EbrAB
Multidrug resistance, efflux pump Bmr
Mce transport system
Manganese/zinc/iron transport system
Manganese/zinc transport system
MalK−MalR (malate transport) two−component regulatory system
LytS−LytR two−component regulatory system
Lipoprotein−releasing system
Lipopolysaccharide transport system
Lipopolysaccharide export system
Lincosamide resistance, efflux pump LmrB
LiaS−LiaR (cell wall stress response) two−component regulatory system
Lantibiotic transport system
L−Cystine transport system
L−Arabinose/lactose transport system
KinABCDE−Spo0FA (sporulation control) two−component regulatory system
Iron(III) transport system
Iron complex transport system
Glycine betaine/proline transport system
Glucose/mannose transport system
GlnK−GlnL (glutamine utilization) two−component regulatory system
General L−amino acid transport system
gamma−Hexachlorocyclohexane transport system
Energy−coupling factor transport system
Dipeptide transport system, Firmicutes
DesK−DesR (membrane lipid fluidity regulation) two−component regulatory system
DegS−DegU (multicellular behavior control) two−component regulatory system
DctS−DctR (C4−dicarboxylate transport) two−component regulatory system
D−Xylose transport system
D−Methionine transport system
Cystine transport system
CssS−CssR (secretion stress response) two−component regulatory system
Competence−related DNA transformation transporter
ComP−ComA (competence) two−component regulatory system
Cobalt/nickel transport system
CitS−CitT (magnesium−citrate transport) two−component regulatory system
CiaH−CiaR two−component regulatory system
CheA−CheYBV (chemotaxis) two−component regulatory system
Cellobiose transport system

Environmental information processing

prop. MAGs [%]



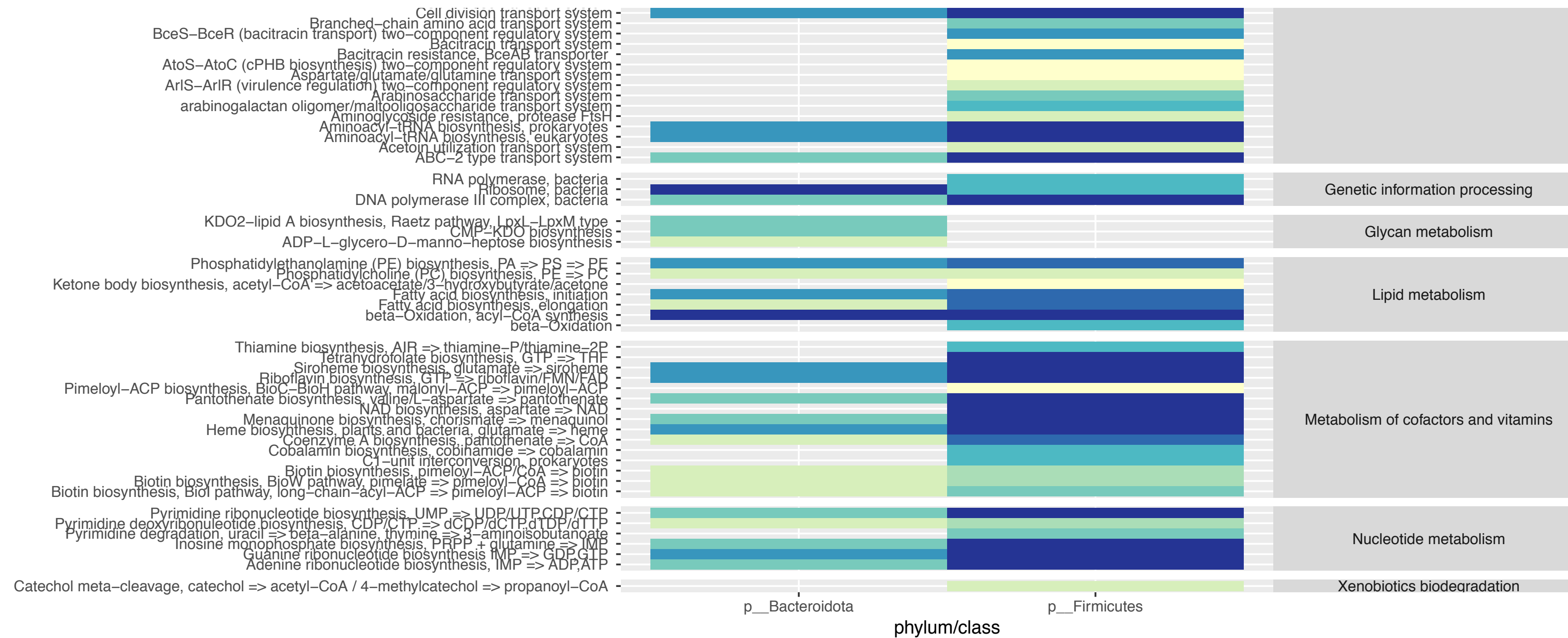
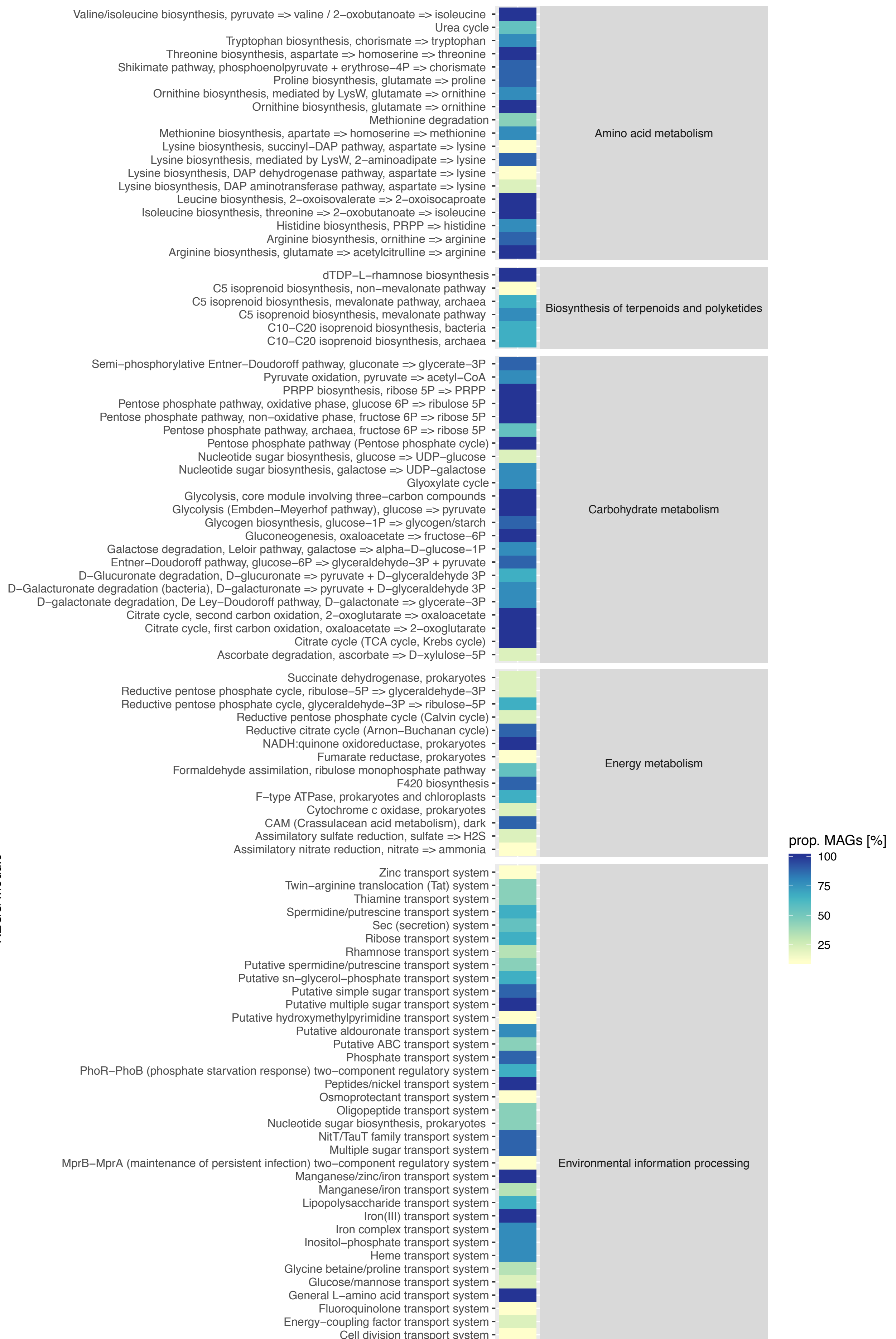


Figure S7.5. KEGG Modules of Bacteroidota and Firmicutes MAGs$_{95\%ANI}$ derived from macroalgae. Colour indicates the number of MAGs$_{95\%ANI}$ (in %) that were associated with individual KEGG Modules


Valine/isoleucine biosynthesis, pyruvate => valine / 2−oxobutanoate => isoleucine
Urea cycle
Tryptophan biosynthesis, chorismate => tryptophan
Threonine biosynthesis, aspartate => homoserine => threonine
Shikimate pathway, phosphoenolpyruvate + erythrose−4P => chorismate
Proline biosynthesis, glutamate => proline
Ornithine biosynthesis, mediated by LysW, glutamate => ornithine
Ornithine biosynthesis, glutamate => ornithine
Methionine degradation
Methionine biosynthesis, apartate => homoserine => methionine
Lysine biosynthesis, succinyl−DAP pathway, aspartate => lysine
Lysine biosynthesis, mediated by LysW, 2−aminoadipate => lysine
Lysine biosynthesis, DAP dehydrogenase pathway, aspartate => lysine
Lysine biosynthesis, DAP aminotransferase pathway, aspartate => lysine
Leucine biosynthesis, 2−oxoisovalerate => 2−oxoisocaproate
Isoleucine biosynthesis, threonine => 2−oxobutanoate => isoleucine
Histidine biosynthesis, PRPP => histidine
Arginine biosynthesis, ornithine => arginine
Arginine biosynthesis, glutamate => acetylcitrulline => arginine
Amino acid metabolism
dTDP−L−rhamnose biosynthesis
C5 isoprenoid biosynthesis, non−mevalonate pathway
C5 isoprenoid biosynthesis, mevalonate pathway, archaea
C5 isoprenoid biosynthesis, mevalonate pathway
C10−C20 isoprenoid biosynthesis, bacteria
C10−C20 isoprenoid biosynthesis, archaea
Biosynthesis of terpenoids and polyketides
Semi−phosphorylative Entner−Doudoroff pathway, gluconate => glycerate−3P
Pyruvate oxidation, pyruvate => acetyl−CoA
PRPP biosynthesis, ribose 5P => PRPP
Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P
Pentose phosphate pathway, non−oxidative phase, fructose 6P => ribose 5P
Pentose phosphate pathway, archaea, fructose 6P => ribose 5P
Pentose phosphate pathway (Pentose phosphate cycle)
Nucleotide sugar biosynthesis, glucose => UDP−glucose
Nucleotide sugar biosynthesis, galactose => UDP−galactose
Glyoxylate cycle
Glycolysis, core module involving three−carbon compounds
Glycolysis (Embden−Meyerhof pathway), glucose => pyruvate
Glycogen biosynthesis, glucose−1P => glycogen/starch
Gluconeogenesis, oxaloacetate => fructose−6P
Galactose degradation, Leloir pathway, galactose => alpha−D−glucose−1P
Entner−Doudoroff pathway, glucose−6P => glyceraldehyde−3P + pyruvate
D−Glucuronate degradation, D−glucuronate => pyruvate + D−glyceraldehyde 3P
D−Galacturonate degradation (bacteria), D−galacturonate => pyruvate + D−glyceraldehyde 3P
D−galactonate degradation, De Ley−Doudoroff pathway, D−galactonate => glycerate−3P
Citrate cycle, second carbon oxidation, 2−oxoglutarate => oxaloacetate
Citrate cycle, first carbon oxidation, oxaloacetate => 2−oxoglutarate
Citrate cycle (TCA cycle, Krebs cycle)
Ascorbate degradation, ascorbate => D−xylulose−5P
Carbohydrate metabolism
Succinate dehydrogenase, prokaryotes
Reductive pentose phosphate cycle, ribulose−5P => glyceraldehyde−3P
Reductive pentose phosphate cycle, glyceraldehyde−3P => ribulose−5P
Reductive pentose phosphate cycle (Calvin cycle)
Reductive citrate cycle (Arnon−Buchanan cycle)
NADH:quinone oxidoreductase, prokaryotes
Fumarate reductase, prokaryotes
Formaldehyde assimilation, ribulose monophosphate pathway
F420 biosynthesis
F−type ATPase, prokaryotes and chloroplasts
Cytochrome c oxidase, prokaryotes
CAM (Crassulacean acid metabolism), dark
Assimilatory sulfate reduction, sulfate => H2S
Assimilatory nitrate reduction, nitrate => ammonia
Energy metabolism
KEGG Module
Zinc transport system
Twin−arginine translocation (Tat) system
Thiamine transport system
Spermidine/putrescine transport system
Sec (secretion) system
Ribose transport system
Rhamnose transport system
Putative spermidine/putrescine transport system
Putative sn−glycerol−phosphate transport system
Putative simple sugar transport system
Putative multiple sugar transport system
Putative hydroxymethylpyrimidine transport system
Putative aldouronate transport system
Putative ABC transport system
Phosphate transport system
PhoR−PhoB (phosphate starvation response) two−component regulatory system
Peptides/nickel transport system
Osmoprotectant transport system
Oligopeptide transport system
Nucleotide sugar biosynthesis, prokaryotes
NitT/TauT family transport system
Multiple sugar transport system
MprB−MprA (maintenance of persistent infection) two−component regulatory system
Manganese/zinc/iron transport system
Manganese/iron transport system
Lipopolysaccharide transport system
Iron(III) transport system
Iron complex transport system
Inositol−phosphate transport system
Heme transport system
Glycine betaine/proline transport system
Glucose/mannose transport system
General L−amino acid transport system
Fluoroquinolone transport system
Energy−coupling factor transport system
Cell division transport system
Environmental information processing
prop. MAGs [%]
100
75
50
25

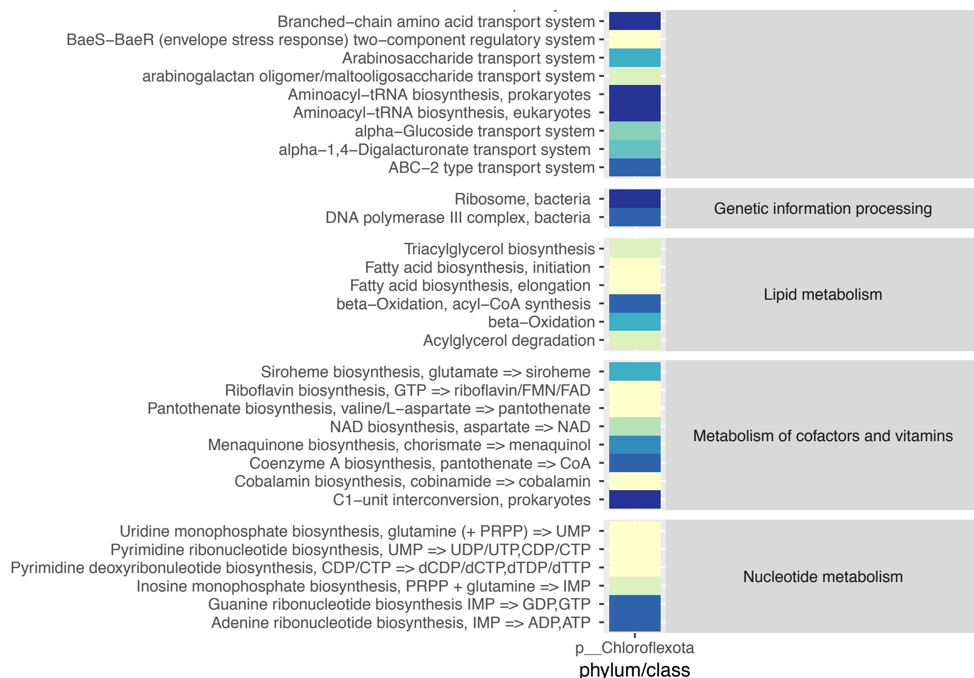


Figure S7.6. KEGG Modules of Chloroflexota MAGs$_{95\%ANI}$ derived from the sponge *Coscinoderma matthewsi*. Colour indicates the number of MAGs$_{95\%ANI}$ (in %) that were associated with individual KEGG Modules.