Corals at the extreme:
partitioning the response of coral holobionts to marginal habitats

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Abstract

While coral reefs worldwide are threatened by unprecedented environmental change, some reef-building corals can already be found living under extreme conditions within marginal habitats. Learning how corals can survive high temperature fluctuations and multiple other stressors experienced in mangroves, relative to typical reefs, is a key step in understanding the adaptive capacity of reef-building corals to future environmental change. The role of the coral host, symbiotic algae, and diverse microbiota, and how these components of the holobiont interact to define the adaptive capacity of reef-building corals requires further exploration. In this thesis, the thermal tolerance limits of conspecific corals from a mangrove versus a reef habitat were tested in a 20-day heat-ramping experiment. Heating corals beyond their regional thermal maxima caused severe decreases in productivity, irrespective of which habitat the coral came from, but corals from the mangrove habitat suffered less thermally induced bleaching. Amplicon sequencing coral holobionts from reef and mangrove habitats in Indonesia and the Seychelles revealed significant habitat-dependent differences in coral microbiome compositions. A potentially novel coral-bacteria symbiosis between a mangrove-dwelling merulinid coral and an unclassified spirochaete, which accounted for 47% of the coral's bacterial community, was also uncovered, though its role in the holobiont remains unknown. Reciprocal translocations of corals between reef and mangrove habitats resulted in rapid reorganisation of coral-associated bacterial communities. Within four days of translocation, coral-associated bacterial communities had changed. Corals demonstrated local adaptation and exhibited increased survivability when back-transplanted in their native habitat than when cross-transplanted to a new habitat. Experimental manipulation of the coral microbiome by antibiotic treatment demonstrated its sensitivity to disturbance, with rapid shifts in bacterial abundance, diversity, and composition taking place within 36 hours. These findings demonstrate the conservation value of mangrove coral habitats and highlight the rapid habitat-dependent flexibility of the coral microbiome.

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My PhD began in the wake of the world's third global mass-bleaching event (colloquially termed the Godzilla El Niño), and ended amidst the ongoing Coronavirus pandemic. Though depressing and stressful at times, working on this four-year long project has taught me so much about resilience (and not just in corals!). I'd like to start by thanking all of my supervisors, including those I started this journey with, and those I gained along the way. Dave, Etienne, Boyd, and Michelle, I really appreciate all the advice and support given, and wouldn't have been able to pull this together without the expertise and encouragement of all of you. You have all helped shape me as a scientist. I would also like to acknowledge the EnvEast Doctoral Training Partnership for support and training provided throughout my PhD, and recognise the scholarship I was awarded from NERC which funded me to conduct this project.

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I dedicate this thesis in loving memory of Jim ‘Papa’ Greenwood.

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Chapter 1: Literature review: Exploring the survival mechanisms of coral holobionts facing sub-optimal environments

NB. Parts of this literature review, ideas, and figures created by Bethan Greenwood, were adapted for publication in the book chapter:

Fry E, Zhu F, **Greenwood B** (2020). Adapting to environmental change. In R. Antwis, X. Harrison, & M. Cox (Eds.), *Microbiomes of Soils, Plants and Animals: An Integrated Approach* (Ecological Reviews, pp. 154-181). Cambridge: Cambridge University Press.

Abstract

Coral reefs worldwide are currently suffering a mass-bleaching crisis. The health status of a coral is determined not only by the host animal, but by its partnership with a diverse assemblage of algal, bacterial, archaeal, fungal, protist, and viral symbionts, collectively termed the coral holobiont. There are corals already existing under the sub-optimal conditions predicted to be experienced by most reefs in the next century. The mechanisms by which these corals are surviving is of great interest and includes short-term, reversible solutions such as phenotypic acclimatisation, long-term Darwinian adaptation, and an intermediate solution whereby corals change their symbionts for more advantageous taxa or strains. Adaptation of holobionts to their surroundings is dependent on their hologenome i.e. the total genetic information of all symbiotic partners. Which microbial associates are essential to all corals, known as the 'core microbiome', and which can be changed dependent on environment, is debated. Key in understanding this will be to determine whether, and how, the microbiome is selected by the coral host. Or is it the case that 'everything is everywhere, but, the environment selects' (Baas Becking, 1934)? Lastly, the

relative contribution of host and microbial partners to the survival of reef-building corals must be established. The answers to these questions have the potential to influence active management interventions such as artificial selection, assisted migration, and probiotic treatment.

1.1. The coral bleaching crisis

Coral reefs concentrate huge biodiversity, estimated between a quarter and a third of the total harboured in marine ecosystems, despite reefs covering less than 0.2% of the ocean surface (Connell, 1978; Reaka-Kudla, 2001). These are under-estimates when considering the vast array of microbiota not counted (Rohwer *et al.*, 2002). This immense biodiversity translates into high productivity, permitting the provision of livelihoods and sustenance for 275 million people living within 30 km of coral reefs worldwide (Burke *et al.*, 2011). Subsequently, reefs are valued at over \$352 000 $ha^{-1}yr^{-1}$ for the goods and services they provide (Costanza *et al.*, 2014).

The scleractinian (reef-building) corals which underpin such highly biodiverse and productive ecosystems are under threat from both anthropogenic and environmental pressures, including over-fishing, pollution, and climate change (Bellwood *et al.*, 2004). Increasing levels of atmospheric carbon dioxide, caused by anthropogenic emissions in the last century (Fig. 1.1 A), have caused a global decrease in ocean pH of 0.1 (Fig. 1.1 D; IPCC, 2007). This phenomenon is known as ocean acidification (Hoegh-Guldberg *et al.*, 2007). Carbon dioxide dissolves into seawater, reacting to produce carbonic acid which dissociates to form bicarbonate and hydrogen ions. These hydrogen ions not only increase acidity, but combine with carbonate ions to produce more bicarbonate ions, thereby reducing the availability of calcium carbonate to calcifying organisms such as coral. Ocean carbonate concentrations have been depleted in this manner by approximately 30 μmol kg^{-1} seawater (IPCC, 2007; Hoegh-Guldberg *et al.*, 2014). Decreasing coral growth rates due to impediment of

calcification by ocean acidification may mean that corals are unable to keep up with the rise in sea levels, also caused by increased greenhouse gas emissions (Fig. 1.1 C).

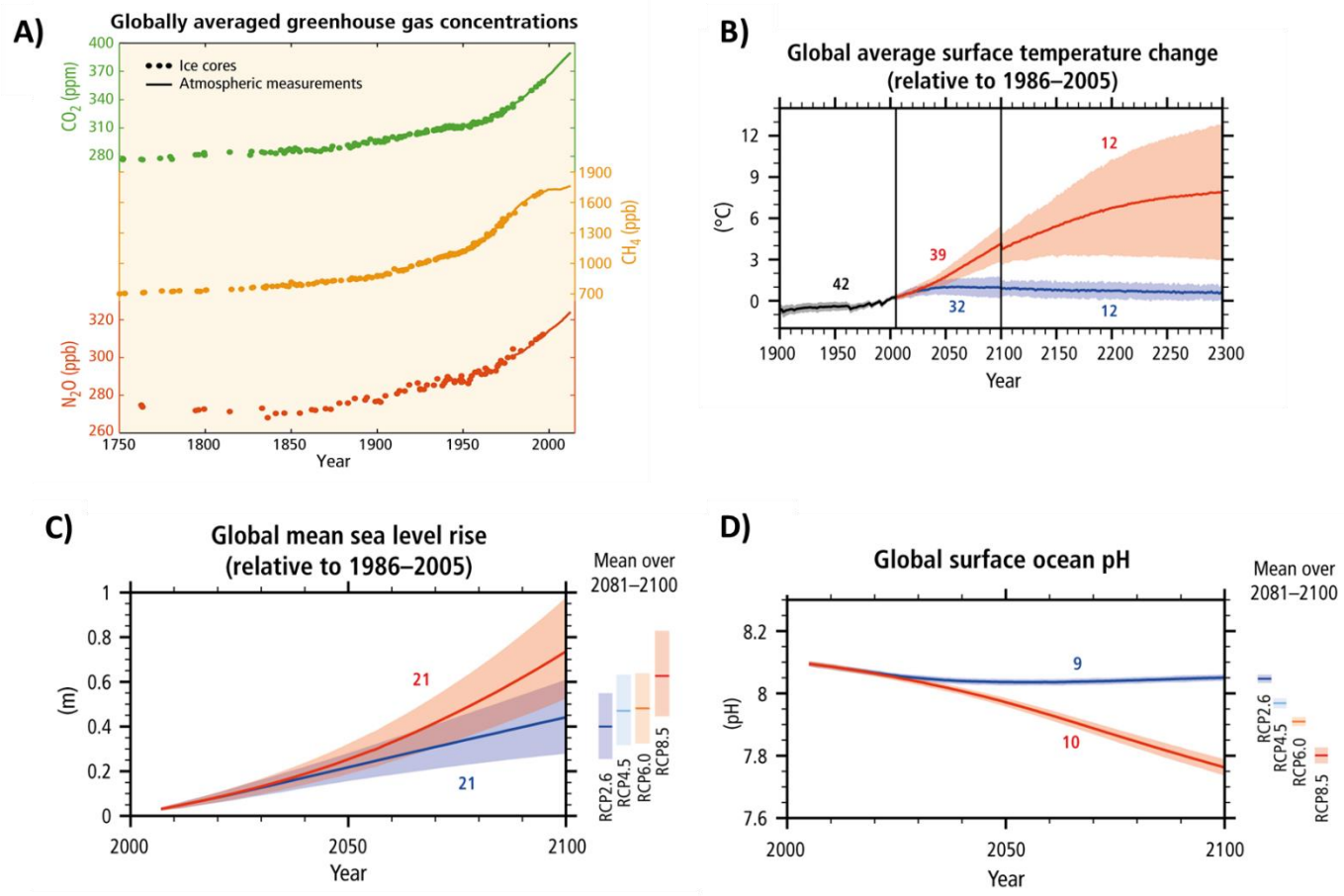

Figure 1.1 A) Increase in greenhouse gas concentrations over the last three centuries. Data from ice cores (circles) with recent direct atmospheric measurements (lines) overlaid. **B)** Time series with projections of global annual change in mean surface temperature. **C)** Global mean sea level rise projections. **D)** Ocean surface pH decrease projections. For panels B-D, all changes are relative to 1986-2005 measurements; time series of predictions (lines) and a 95% measure of uncertainty (shading) are shown for best case (blue) and worst case scenarios (red). The number of models used to calculate mean projections is indicated (From IPCC AR5 report, 2013).

Shallow-water reef-building corals are currently living close to their upper thermal limits (Berkelmans & Willis, 1999) and are restricted to the uppermost layer of low-latitude oceans as they rely on harnessing energy from the sun to grow (Yellowlees *et al.*, 2008). Global warming due to greenhouse gas emissions is unequivocal and significant warming has occurred in the oceans' surface since the start of the 20th century (IPCC, 2013). The Intergovernmental Panel on Climate Change (IPCC) predict that continuation of the current greenhouse gas emissions will lead to an increase in tropical sea surface temperatures

(SSTs) of 3-4˚C, by 2100 (Fig. 1.1 B; Hoegh-Guldberg *et al.*, 2014; ISRS, 2015). When SSTs exceed thermal thresholds for sustained periods, coral bleaching occurs. Corals appear 'bleached' due to the breakdown in relationship between colourless coral polyps and pigmented microalgae which reveals the white carbonate skeleton underneath the transparent coral tissue (Brown, 1997). Prolonged periods of elevated SSTs lead to mass coral bleaching; episodes of which have become more frequent and severe. The most comprehensive satellite-based study of SST has recently shown that bleaching-level thermal stress has increased three-fold in the last three decades with 97% of reef areas experiencing warming (Heron *et al.*, 2016).

The threat of coral bleaching due to SST anomalies is monitored by the National Oceanic and Atmospheric Administration (NOAA), who implement a measure of accumulated thermal stress above the local average summer maximum SST, known as degree heating weeks (DHW) (Fig. 1.2). The measure assesses the likelihood of bleaching based on both the intensity and duration of an anomalous elevated SST event. Since mass bleaching events are caused by prolonged periods of thermal stress, the DHW measure accumulates occurrences of SSTs greater than 1°C above the local average temperature of the hottest month, for the past 12 weeks. This determines how much thermal stress corals have undergone in the last three months.

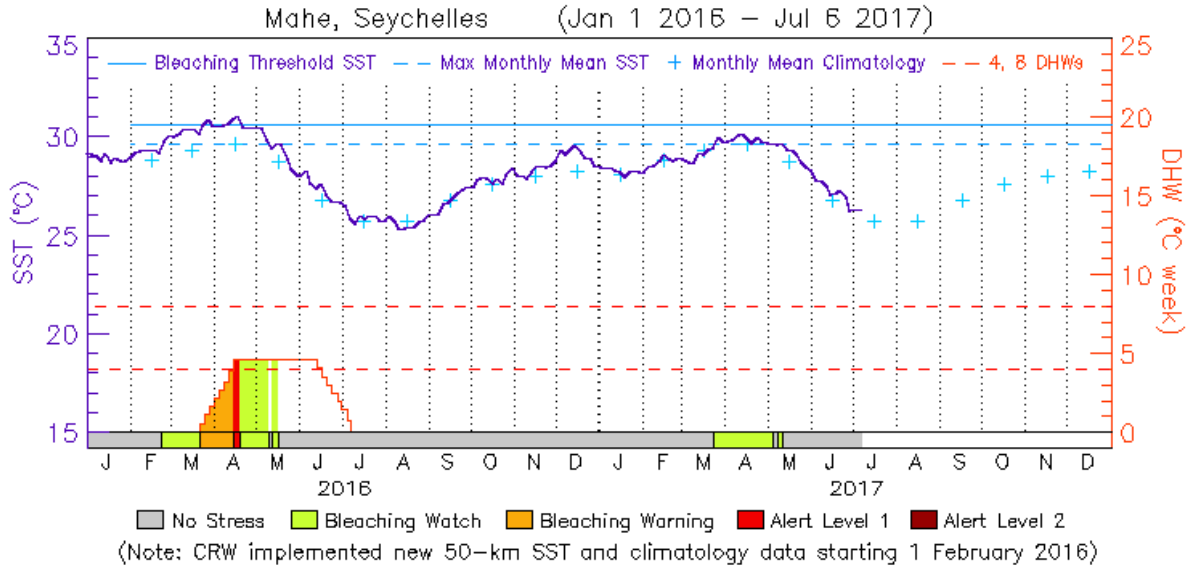

Figure 1.2. Coral Reef Watch two-year time series graph for Mahe, Seychelles. Sea surface temperatures (purple line) use left vertical axis; Degree Heating Week (DHW) (red line) use right vertical axis; Bleaching Alert Levels are shaded under the DHW line and correspond to the legend underneath the horizontal axis. Typical local SST for each month is shown (Monthly Mean Climatology as light blue crosses). The Bleaching Threshold (light blue solid line) for an area is 1°C above the local average summer maximum (Maximum Monthly Mean SST shown as light blue dashed line). The threshold for Bleaching Alert Level 1 is 4°C-weeks when significant bleaching is expected (red dashed line). The threshold for Alert Level 2 is 8°C-weeks (red dashed line) when mass-bleaching is expected.

Amid the recent 'monster El Niño', corals appeared poorly adapted to even 1°C rises in temperature above their usual summer maximum (Cressey, 2016). Many taxa have responded to rapid climate change by shifting their range, but since corals are sessile organisms, they cannot escape unfavourable conditions. Due to their longevity, traditional Darwinian adaptation over generational time is not fast enough, so corals must find other ways to survive a changing climate or risk extinction (Carpenter *et al.*, 2008).

Box 1.1. Mechanisms for coral survival

Resistance – the ability to withstand stress (coral species lie on a continuum from susceptible to bleaching-resistant).

Resilience – the capacity for recovery of either an individual (e.g. whether a colony recovers from bleaching) or a community (e.g. whether a reef can remain coral-dominated or shifts to an alternative state).

Acclimation – the adjustment of an organism to a change in laboratory environment, whereby it becomes accustomed to artificially induced conditions.

Acclimatisation – an experience-mediated increase in resistance (to bleaching), referring to environmentally inducible phenotypic traits.

Adaptation – an evolutionary process, referring to the inheritance of genotypic traits, that have evolved through natural selection.

Despite this gloomy outlook, there are already corals surviving beyond what were thought to be their limits in marginal habitats (Kleypas *et al.*, 1999). Mangroves and seagrass beds harbour corals with the ability to grow under lower than optimal pH and aragonite saturation and withstand continually fluctuating temperature and light conditions (Yates *et al.*, 2014). Researchers can use extreme or marginal habitats as 'natural laboratories' to predict how corals will respond to climate change. Understanding how corals can survive current sub-optimal conditions may provide a forecast for the future of coral reefs and help us ensure their continued provision of ecosystem goods and services.

The aim of this literature review is to outline the mechanisms (Box 1.1) by which corals can survive imminent environmental change toward sub-optimal conditions. These mechanisms will be explored via the various components of the 'coral holobiont'.

1.2. The coral holobiont

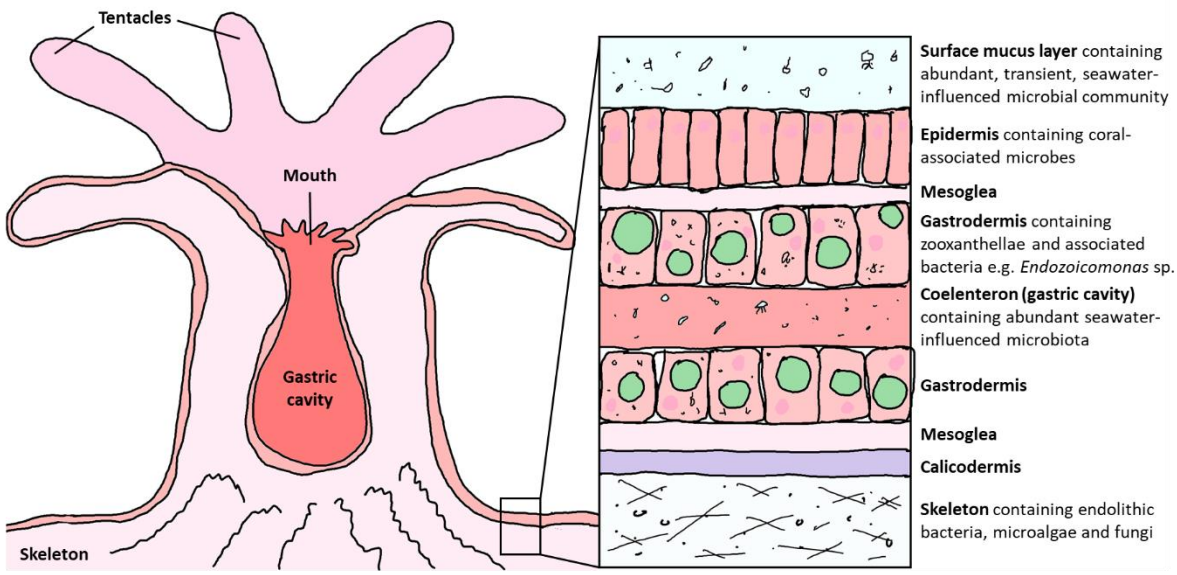

Figure 1.3. A schematic section of a coral polyp showing coral holobiont symbionts associated with various compartments: surface mucus layer (SML), epidermis, mesoglea, gastrodermis, gastric cavity, calicodermis and skeleton (illustration: Bethan Greenwood, adapted from Bourne *et al.*, 2016; now published in Fry *et al.*, 2020).

The term ‘coral holobiont’ was coined by Rohwer *et al.* (2002) to define the meta-organism consisting of host cnidarian, symbiotic zooxanthellae and other microbial associates (Fig. 1.3). With their symbiotic and parasitic microorganisms, which by far surpass the number of host cells, virtually all organisms can be considered meta-organisms (Bosch & McFall-Ngai, 2011). There has been a recent movement in evolutionary biology toward viewing all systems as holobionts (Rosenberg & Zilber-Rosenberg, 2016). The most well-documented example of such is the human holobiont. The Human Microbiome Project focussed on sequencing every symbiotic microorganism on and in the human body (The Human Microbiome Project Consortium, 2012). One of the main findings was that of remarkable functional stability despite large variation in taxonomic composition of different human microbiomes, suggesting functional diversity may be more important than taxonomy. In other well-studied holobionts, microbial symbionts are known to perform roles which the host is incapable of otherwise e.g. cellulose digestion in ruminants (Russell *et al.*, 2009) and nitrogen fixation in legume roots (Oldroyd *et al.*, 2011).

In coral holobionts, photosynthesis is carried out by microscopic dinoflagellate algae, called zooxanthellae, enabling the coral to supplement its diet with an extra carbon source (Yellowlees *et al*., 2008; Fig. 1.4). Bacteria within coral tissues have been purported to play roles in nitrogen and sulphur cycling, metabolising otherwise unavailable nutrients (Rohwer *et al*., 2002). Without these symbiotic associations, corals could not survive in such clear, nutrient-deficient water as those found around reefs (see Darwin's Paradox, 1842). Due to the functional importance of these symbioses, there is likely a coral host-mediated immune response for selecting beneficial microbes, while rejecting pathogens (Krediet *et al*., 2013).

Since the coral holobiont functions together as one entity, the collective DNA and RNA of the host and all its symbionts can be viewed as a unit for selection to act upon (Rosenberg *et al.*, 2007). This forms the basis of the 'hologenome theory of evolution' (Zilber-Rosenberg & Rosenberg, 2008), whereby adaptation and evolution of meta-organisms relies upon the enormous genetic diversity bestowed by the microbial symbionts. Since members of the microbiome have short generation times, they can adapt to new environmental regimes faster than their higher-organism hosts; thus presenting a potential mechanism for corals to keep up with rapid climate change. Counter-arguments to the hologenome, however, describe a host plus its microbiome as an ecological community encompassing 1) a range of symbiotic interactions (from parasitic to mutualistic), 2) differing levels of host-microbe fidelity, as well as 3) conflicting fitness interests between microbial constituents – meaning that the whole community cannot evolve as one unit (Douglas & Werren, 2016).

Should a holobiont survive stressful conditions, it would be advantageous for the associated microbiome to be inherited by future generations to maintain stress-tolerant properties. However, vertical transmission has been rejected as a mode of bacterial transfer in some corals (Apprill *et al.*, 2009). An investigation into the microbiomes of lab-reared deer mice, *Drosophila* flies, mosquitoes, and wasps recently demonstrated that the more closely phylogenetically- (and therefore evolutionarily-) related host species are, the more similar

their microbiomes (Brooks *et al.*, 2016). This may indicate that there has been selection to maintain host-microbe relationships over evolutionary time, which the authors termed 'phylosymbiosis'. To test this theory, microbiome transplants were conducted between species of both *Peromyscus* deer mice and *Nasonia* wasps, which revealed even closely related species' microbiomes to be less functional than the hosts' original.

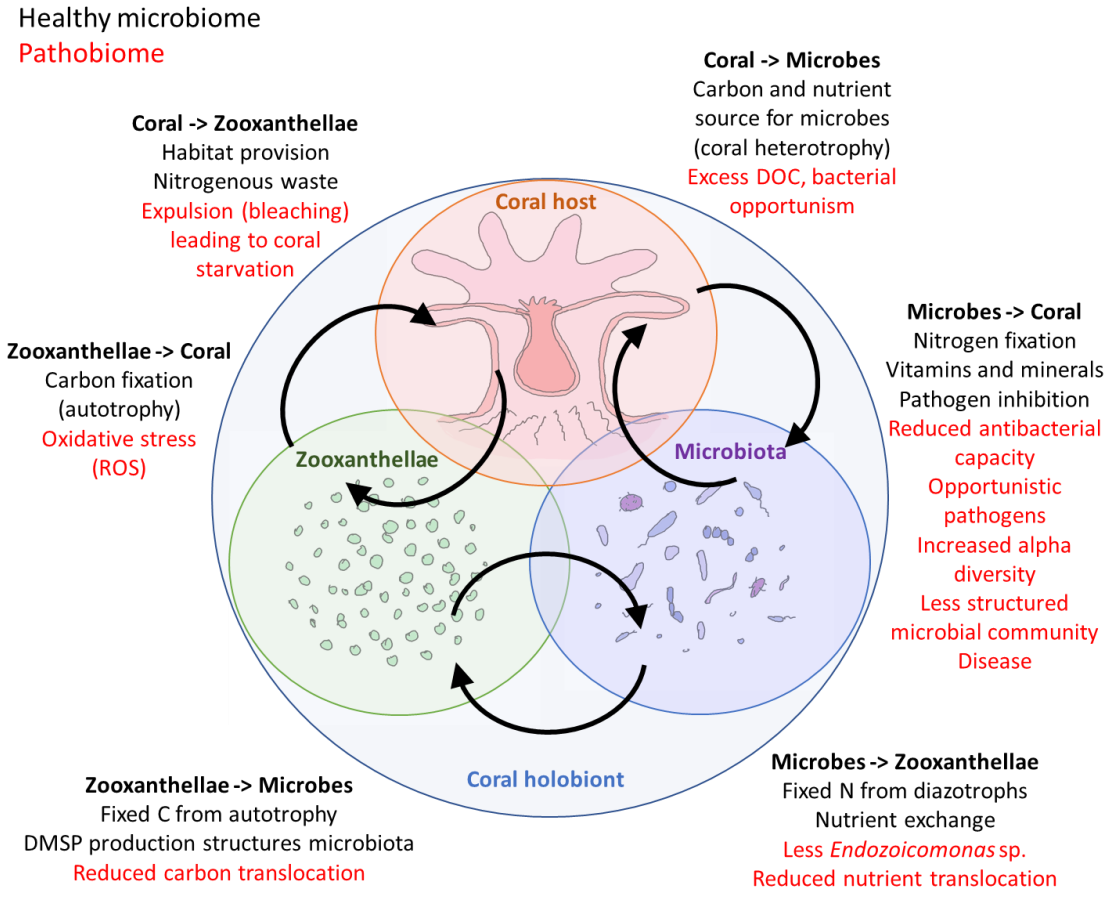

Figure 1.4. Conceptual figure showing purported roles of the coral host, and associated microbiome, including both zooxanthellae and other microbiota, within the coral holobiont. Functions of a healthy microbiome shown as black text, and impaired functions due to a disease-associated microbiome or 'pathobiome' (*sensu* Sweet & Bulling, 2017) during times of environmental stress as red text. (Illustration: Bethan Greenwood, adapted from Vega Thurber *et al.*, 2009; now published in Fry *et al.*, 2020).

If the coral microbiome is inherited through vertical transfer, this should be reflected in stable, species-specific coral microbiomes. Several studies have provided evidence for coral host-specific microbial communities (Rohwer *et al.*, 2002; Littman *et al.*, 2009; Kvennefors *et al.*, 2010). However, coral-associated microbial communities have also been shown to be

influenced by biogeography (McKew *et al.*, 2012), physical environment (Littman *et al.*, 2009), and season (Koren & Rosenberg, 2006) as well as partitioning of bacterial communities between the various compartments of corals, including the mucus, tissue, and skeleton (Fig. 1.3; Sweet *et al.*, 2011a). The notion of a 'core microbiome' – a set of microbiota associated with all individuals of a host species (Shade & Handelsman, 2012) – is a relatively new concept in coral microbiology, therefore, what is defined as 'core' varies across studies. Ainsworth *et al.* (2015) considered presence of a microbial phylotype in at least 30% of 454-sequenced coral samples to represent a member of the core microbiome, whereas Hernandez-Agreda *et al.* (2016) considered a cut-off of 80% presence across Illumina-sequenced corals to represent the core microbiome. Cut-offs as high as 100% have been reported for the gorgonian coral, *Corallium rubrum* (van de Water *et al.*, 2016). From these studies of core coral microbiomes, it has emerged, similar to the Human Microbiome Project Consortium (2012), that there are very few (seven acknowledged by Ainsworth *et al.* (2015) and eight included by Hernandez-Agreda *et al.* (2016)) core microbiome members shared across all coral host species. This suggests that providing functional diversity is maintained, it does not matter which taxa are present. Another key finding was that low-abundance microbiota can form important stable relationships with their host (Ainsworth *et al.*, 2015). Many studies focus on the most abundant OTUs, and neglect taxa which despite appearing rare within a holobiont, could provide a key function and be persistent among different spatial and temporal scales. These highly conserved microbes are likely the ones which play important roles in their host's fitness.

1.3. The coral host

1.3.1. Host genotype

The cnidarian host's genetic material is only part of the genetic bank from which the coral holobiont can adapt to sub-optimal conditions. Certain coral genera, such as *Porites* spp.,

are commonly defined as being more stress-tolerant than others, with coral taxa often viewed as lying on a continuum of bleaching susceptibility (Loya *et al.*, 2001). For every species, there are likely hard limits to their resistance capabilities, but within this window exists a large degree of phenotypic variation dependent on environment and thermal history. Natural variation in the gene expression of coral hosts within and between populations has been studied extensively in attempts to explain different acclimatisation potential.

Kenkel *et al.* (2013) revealed, through microsatellite genotyping, that the coral host was responsible for differences in thermotolerance of *Porites astreoides* in the Florida Reef Tract. Corals from a more temperature-variable inshore reef, and less variable offshore reef, were subjected to a 6-week temperature stress of 31°C in a common-garden experiment. Corals from inshore reef showed significantly less bleaching and increased growth compared with corals from the offshore reef, despite no significant difference in algal symbiont haplotype frequency or symbiont shuffling in response to thermal stress. Genetic divergence detected between coral host populations and differences in host metabolism between locations (Kenkel *et al.*, 2013b) strongly suggested a host role in coral holobiont thermotolerance. To determine whether the thermotolerance differences between these coral populations were due to heritable genetic variation or long-term acclimatisation to their inshore/offshore environment, Kenkel *et al.* (2015) then used naïve juvenile corals from parental colonies from inshore vs offshore environments to minimise any influence of prior acclimatisation to different habitats. These juvenile corals were reared in a common aquarium for 5 weeks to minimise any maternal effects, before subjecting the recruits to either a thermal stress of 31°C or a control of 28°C for 2.5 weeks. While there was no mortality due to heat stress, inshore-origin recruits grew significantly more under thermal treatment compared with offshore recruits. The authors therefore concluded that host population-level fitness variation in response to elevated temperature has a genetic basis and thus could represent a means for natural selection to act upon during climate change. In agreement, Dixon *et al.* (2015) showed a nearly 10-fold increase in survival probability of coral larvae under heat stress if

their parent colonies came from a warmer low-latitude location. This increased thermal tolerance coincided with inherited differences in gene expression for oxidative, extracellular, transmembrane transport, and mitochondrial functions. This supports the idea that thermal tolerance is heritable and thus corals could avoid extinction via 'genetic rescue' i.e. spatial transfer of advantageous tolerant genotypes.

The Persian-Arabian Gulf (PAG) is recognised as an extreme environment for corals as it reaches temperatures of 36°C in summer, representing end-of-century temperature projections for coral reefs worldwide. This 'natural laboratory' has prompted research into the genetic adaptation needed to cope with extreme PAG temperatures. *Platygyra daedalea* corals from the PAG have unsurprisingly been shown to exhibit increased thermotolerance when compared with their conspecifics from the milder Sea of Oman (Howells *et al.*, 2016). Survivability at 36°C of both asymbiotic larvae and symbiotic adults, was higher in PAG corals, and PAG hosts were able to mitigate oxidative stress better, supporting a host role in thermotolerance. Even after 6 months acclimation at a common ambient environment, PAG corals exhibited superior thermotolerance, supported by the detection of genetic divergence in the host and zooxanthellae (Howells *et al.*, 2016).

Coral thermotolerance is a complex or polygenic trait, i.e., it is governed by many different genes (Thomas *et al.*, 2018). Studies of corals from highly thermally-variable back-reef pools on Ofu Island, American Samoa, revealed that a number of alleles across different cellular pathways were responsible for elevated thermal tolerance (Bay & Palumbi, 2014; Palumbi *et al.*, 2014). Heat resistance in corals is further complicated since resistance to bleaching under short-term heat shocks is not always a reliable predictor of resistance to prolonged heating events (Morikawa & Palumbi, 2019). This highlights that there are different strategies or traits needed for surviving different types of thermal stress (e.g. front-loading of genes combats acute thermal stress; Barshis *et al.*, 2013), but also that thermotolerance is the

result of a combination of many different components including host genotype, symbiont types, thermal history, thermal microclimate etc.

1.3.2. Host acclimatisation

Other survival mechanisms of coral hosts during thermal stress are genotype-independent and rely solely on phenotypic change. *Acropora millepora* from reef flats of the Great Barrier Reef has been shown to increase its resistance to thermally-induced bleaching without any changes in zooxanthellae or bacterial symbiont composition following short-term (10-day) laboratory acclimation at 3°C below the bleaching threshold (Bellantuono *et al.*, 2011). The authors concluded that phenotypic plasticity in the coral host's physiology was important in rapid temperature acclimation.

Back-reef and tidal pools in American Samoa exhibiting different temperature regimes provide a 'natural laboratory' for reciprocal transplant experiments. These experiments can disentangle host colony effects from environmental influences on coral response to extreme environments. When *Porites lobata* was transplanted from a stable-temperature fore-reef in Samoa to a neighbouring back-reef which experiences large daily temperature fluctuations, Barshis *et al.*, (2010) found there to be both fixed genetic and environmental influences on biomarker response. Source colony identity had greater influence than transplant environment on ubiquitin-conjugated protein levels – a biomarker for stress resistance - and therefore the authors hypothesised that the host genotype had limited phenotypic plasticity. This was supported by indistinguishable algal symbiont populations hosted by corals from back and fore-reefs, but genetic differentiation between coral host populations. While there was some effect of transplant environment on biomarker response, representing acclimatisation, the strong influence of colony origin suggested that coral populations may be limited in their physiological capacity to respond to new stressful conditions.

Other studies from this 'natural laboratory' showed corals from more temperature-variable pools exhibited greater thermal tolerance (Oliver & Palumbi, 2011a), faster growth rates (Smith *et al.*, 2007), and more thermotolerant algal symbiont genotypes (Oliver & Palumbi, 2011b), in addition to the higher aforementioned protein biomarker levels (Barshis *et al.*, 2010), than conspecific corals from thermally stable tide pools. Bay and Palumbi (2014) subsequently claimed that corals from naturally high temperature variation pools were less bleaching-susceptible due to both acclimatisation and fixed genetics. By genotyping 15,399 single-nucleotide polymorphisms from 23 *Acropora hyacinthus* colonies from different pools, they found that corals from the warmest environments had the highest number of minor allele frequencies. They concluded that this natural population possessed a reservoir of alleles pre-adapted to high temperatures.

Similarly, acclimatisation to highly variable pH environments has been claimed to enhance resistance to the effects of ocean acidification (Comeau *et al.*, 2014). However, Camp *et al.* (2016) found that Caribbean corals from highly pH- and temperature-variable seagrass beds and less variable neighbouring reef showed no difference in their calcification ability when subjected to current-day as well as predicted year-2100 high variation temperature and pH conditions. This suggests that marginal habitats may not harbour corals pre-adapted to, nor act as refugia against, future climate change.

1.3.3. Gene expression

Genomic investigations have shown just how vital the coral host is in responding to stress, via the up- and down-regulation of genes coding for defences such as heat shock proteins (HSPs; Brown *et al.*, 2002), antioxidants (Brown *et al.*, 2002; Barshis *et al.*, 2010), and those involved in changes in cell adhesion and apoptosis initiation (Ainsworth & Hoegh-Guldberg, 2008; Barshis *et al.*, 2010, Barshis *et al*., 2013; Bellantuono *et al.*, 2011). Other defences

provided specifically by the coral host include green fluorescent proteins (GFPs) and other fluorescent pigments (Salih *et al.*, 2000).

Heat shock proteins are a family of proteins, including many molecular chaperones, which play important roles in cellular repair and maintenance of protein structural integrity during stressful conditions (Arya *et al.*, 2007). Brown *et al.*, (2002) demonstrated the importance of *Coelastrea aspera* (previously known as *Goniastrea*) host tissues in preventing thermally-induced bleaching under high light by their increased HSP 60 and 70 levels, without any sign of algal symbiont defences. Fluorescent pigments play a photoprotective role by absorbing, scattering, and dissipating damaging radiation (Salih *et al.*, 2000). The concentration of fluorescent pigments in host tissue has been strongly correlated to bleaching resistance for 21 Great Barrier Reef coral species (Salih *et al.*, 2000). The capacity of a host to produce antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase, to detoxify reactive oxygen species, influences the holobiont's ability to resist bleaching (Brown *et al.*, 2002; Barshis *et al.*, 2010)**.** Several studies have shown up-regulation of genes involved in antioxidant production in response to stress. For example, *Acropora millepora* has been shown to up-regulate catalase genes during natural bleaching events (Seneca *et al.*, 2010). Other non-enzymatic antioxidants reportedly produced by corals include ascorbic acid (vitamin C), tocopherol (vitamin E), glutathione, carotenoids, dimethylsulfide (DMS) and mycosporine-like amino acids (MAAs) (Lesser, 2006).

Following long-term acclimatisation to different thermal regimes in tidal pools, *Acropora hyacinthus* were exposed to simulated thermal bleaching stress in the laboratory (Palumbi *et al.*, 2014). Surprisingly, during thermal stress, the more thermotolerant corals from high temperature-variation pools exhibited less up-regulation of genes related to heat tolerance than the more sensitive corals from low temperature-variation pools (Barshis *et al.*, 2013). It transpired that these genes, including those which code for heat shock proteins (HSPs) and antioxidant enzymes involved in heat tolerance, as well as some involved in apoptosis

regulation, tumour suppression, immune response and cell adhesion, were transcribed constantly (even under ambient temperature) in corals which frequently experience large temperature fluctuations. This front-loading in gene expression may promote coral resistance to frequently encountered stress.

The energy demands for production of HSPs and antioxidants are very high, thus acclimation and acclimatisation are energetically costly (Brown, 1997). Resilient coral hosts can survive bleaching by up-regulating heterotrophy, thereby gaining enough energy from zooplankton consumption (Grottoli *et al.*, 2006), or by utilising their lipid stores to avoid starvation (Rodrigues & Grottoli, 2007). It should be noted that most studies on gene expression in response to thermal stress have been the result of short-term heating experiments in the laboratory (a summary of such studies can be found in Sweet & Brown, 2016).

1.3.4. Host control over symbionts

A huge current question in coral biology exists around the role of the host in regulating microbial diversity and maintaining the stability of the coral holobiont. Since corals depend partly on their microbial symbionts for functions they are unable to perform (see Fig. 1.4), and may become more reliant on these during periods of stress, it is important to discover how the host influences the composition and functions of its microbial partners. Coral hosts must either be able to detect and differentiate microorganisms to select for beneficial partners while defending against undesirable microbes, or they must excrete broad-spectrum antimicrobial compounds to select against environmental organisms (Krediet *et al.*, 2013). There is more evidence for the latter scenario as antimicrobial compounds have been found from *Siderastrea siderea* (Gochfeld *et al.*, 2006), *Montipora captitata*, *Porites lobata*, and *Pocillopora meandrina* (Gochfeld & Aeby, 2008). These mechanisms suggest that the composition of microbiota is important (Krediet *et al.*, 2013). Whereas other theories

suppose that obtaining and maintaining functions are more important, for example if the coral host produced specific chemical cues to attract microbes with beneficial functions or with the ability to shape the microbial community and prevent pathogen invasion (Wegley *et al.*, 2007; Krediet *et al.*, 2013). These theories are not supported by a wealth of evidence but have been modelled in regulating coral disease development (Mao-Jones *et al.*, 2010). The coral host may also play a role in choosing to release its symbiotic algae as an immune-like response during periods of stress, by host production of nitric oxide as a cell-death inducing signal (Weis, 2008).

The degree to which corals can acclimatise to sub-optimal conditions with regular exposure depends partly on the host's phenotypic plasticity, but hard limits are ultimately determined by the host's genetic material (genotype). There is hope that the adaptive limits of coral holobionts can be expanded with help from symbionts providing functions which the coral host is unable to.

1.4. Zooxanthellae

1.4.1. What are they? Their symbiotic relationship with coral

Zooxanthellae are microscopic, single-celled dinoflagellates capable of photosynthesis. It is this trait which underpins their symbiotic relationship with coral. In a fully functioning symbiosis, the coral host benefits from provision of up to 90% of its energy requirements in the form of autotrophically-fixed organic carbon (Muscatine, 1990; Yellowlees *et al.*, 2008). In exchange, the zooxanthellae receive carbon dioxide, essential nutrients, and trace elements which are otherwise scarce in the open ocean, and a refuge beneath transparent coral tissues with access to sunlight (Fig. 1.4).

1.4.2. The breakdown of the relationship – bleaching

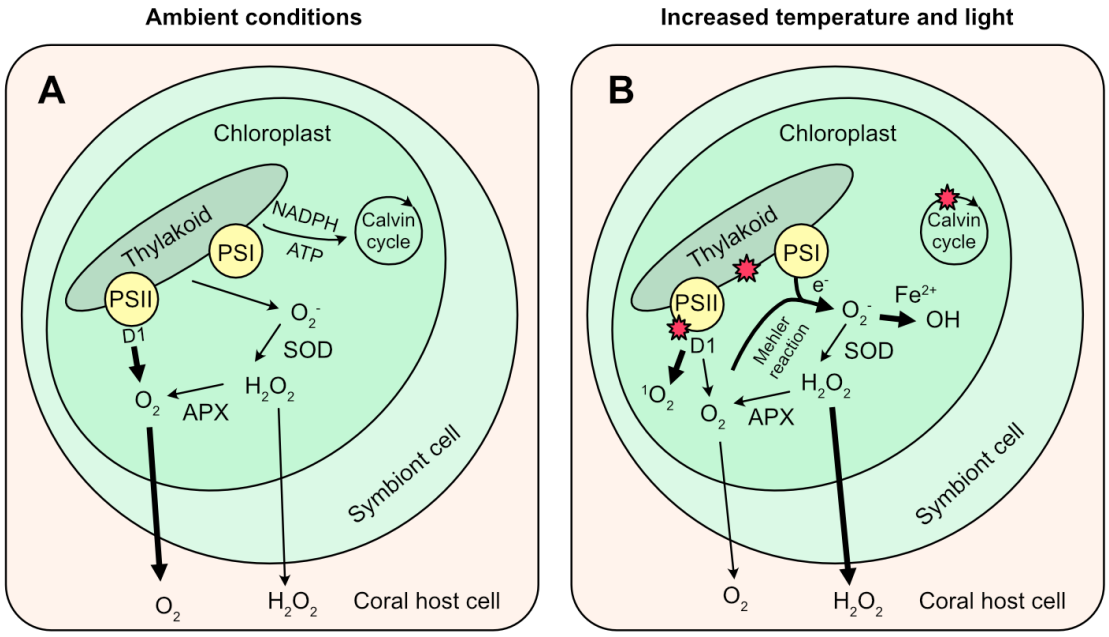

Figure 1.5. Schematic diagram of the symbiotic relationship between zooxanthellae and coral host tissue **A)** under ambient conditions, where photosystem I (PSI) and photosystem II (PSII) operate as normal, producing large amounts of oxygen which diffuse to the host. The antioxidant enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) convert low levels of reactive oxygen species (ROS) back into oxygen. **B)** During the breakdown in relationship due to elevated light and/or temperature conditions, damage occurs to the photosynthetic apparatus (PSI and PSII; red flashes in diagram) causing the generation of unusually high levels of ROS, such as superoxide (O_2^-). These overwhelm the oxygen-handling pathways and accumulate as they are not detoxified. Superoxide is then converted to the most reactive ROS, hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) which cause damage in the zooxanthellae and host cells (adapted from Weis, 2008).

However, the breakdown of this symbiotic relationship can occur due to stress induced by environmental changes, notably temperature and light. Like all oxygenic photosynthetic organisms, zooxanthellae risk photo-oxidative damage (Roth, 2014). Accumulation of reactive oxygen species (ROS), due to cascading effects of impaired photosynthetic apparatus and continued oxygen production, leads to oxidative stress, resulting in cellular damage to membranes, proteins and nucleic acids (Lesser, 2006). It is thought that the compromised and damage-causing zooxanthellae are either ejected (exocytosis), eliminated (apoptosis), or eaten (phagocytosis) by the host as an innate immune response, resulting in coral bleaching (Weis, 2008). However, it should be noted that not all bleaching occurs

because of a breakdown in symbiosis. There is a wealth of literature on bleaching mechanisms alone (reviewed in Fitt *et al.*, 2001); others include sub-lethal paling of zooxanthellae and natural seasonal changes in pigmentation (Suggett & Smith, 2011).

1.4.3. Symbiodiniaceae systematics and thermotolerance

All zooxanthellae which inhabit coral tissues belong to the family Symbiodiniaceae (LaJeunesse *et al.*, 2018). Originally, there was thought to be only one species, now known as *Symbiodinium microadriaticum* Freudenthal, 1962 (Taylor, 1971). The advent of molecular systematics and polymerase chain reaction (PCR)-based technology allowed identification of several clades of Symbiodiniaceae (Rowan & Powers, 1991) with differing physiological limits (Kinzie *et al.*, 2001; Rowan, 2004; Baker & Romanski, 2007). These 'clades' have now been re-classified as separate genera (LaJeunesse *et al.*, 2018 – See Box 1.2). Rowan (2004) observed that *Pocillopora* spp. in Guam seemed to have differing temperature tolerances dependent on which genus of Symbiodiniaceae was hosted. When *Pocillopora damnicornis* and *P. verrucosa* were subjected to increased temperatures in the laboratory, the photophysiology of corals hosting *Cladocopium* (formerly Clade C) vs *Durusdinium* (formerly Clade D) was significantly different. For corals hosting *Cladocopium*, Fv/Fm and productivity:respiration ratio was significantly decreased compared with control

Box 1.2. New genus-level taxonomic classifications of Symbiodiniaceae (LaJeunesse *et al.*, 2018; Nitschke *et al.*, 2020)

Symbiodinium Gert Hansen & Daugbjerg 2009 (Freudenthal, 1962 attribution was deemed invalid under ICN Article 40.6 as no type specimen was collected) – formerly **Clade A**.

Breviolum J.E.Parkinson & LaJeunesse – formerly **Clade B**.

Cladocopium LaJeunesse & H.J.Jeong – formerly **Clade C**.

Durusdinium LaJeunesse – formerly **Clade D**.

Effrenium LaJeunesse & H.J.Jeong – formerly **Clade E**.

Fugacium LaJeunesse – formerly **Clade F**.

Freudenthalidium Nitschke & Craveiro – formerly **Clade Fr3**.

Gerakladium LaJeunesse – formerly **Clade G**.

Halluxium Nitschke & Craveiro – formerly **Clade H**.

thermal treatments, while for those hosting *Durusdinium*, photophysiology either increased or remained the same with increased temperature. The discovery that corals could host more than one clade of Symbiodiniaceae (Rowan *et al.*, 1997; Rowan, 2004) gave rise to the theory that corals could change their algal symbionts over space and time.

1.4.4. Adaptive bleaching hypothesis

The 'adaptive bleaching hypothesis' (ABH) theorises that coral bleaching provides an opportunity for repopulation by more beneficial Symbiodiniaceae from the local environment (Buddemeier & Fautin, 1993). Baker *et al.* (2004) carried out molecular surveys of Symbiodiniaceae across the globe following the 1998 El Niño. They found that corals associated with *Durusdinium* were more abundant on reefs which had suffered severe mass-bleaching events, and that coral- Symbiodiniaceae associations on previously severely affected reefs closely resembled those found in naturally elevated temperature environments such as the Persian-Arabian Gulf (PAG); thus pointing to a SST-induced adaptive shift in symbionts toward thermal tolerance. Interestingly, the PAG harbours corals able to withstand remarkably high salinities and temperatures exceeding 35°C, which has been partly explained by their association with a newly discovered symbiont – *Cladocopium thermophilum* (ITS2 type C3) (D'Angelo *et al.*, 2015). Based on its phylogeography, researchers believe that this stress-tolerant symbiont came from a large, diverse ancestral group of Symbiodiniaceae*,* which are now barely detectable outside the PAG (Hume *et al.*, 2016). The authors suggest it was naturally selected by extreme temperatures in the Holocene, emphasising the importance of Symbiodiniaceae genetic diversity for future climate change selection to act upon.

Although there has been a lot of attention on the promise of the ABH for rapid adaptation to fast-paced climate change, a consensus has not yet been reached. The main argument centres around how the Symbiodiniaceae community within a coral holobiont shifts. Some

adult corals have been shown to uptake Symbiodiniaceae from the environment (Lewis & Coffroth, 2004), known as ‘symbiont switching’, but whether the new associations remain stable or the new symbionts are able to become dominant in the holobiont is unknown. Coral hosts also seem to exhibit high fidelity to certain clades (Goulet, 2006; Rodriguez-Lanetty *et al.*, 2004; Sampayo *et al.*, 2016) and therefore changes in the relative abundance of existing symbionts, known as ‘symbiont shuffling’ may be the more prevalent mechanism of symbiont change.

1.4.5. Symbiont switching

Baker (2001) conducted reciprocal transplantations of eight Caribbean coral species between shallow and deep sites in Panama to investigate the ABH. Corals transplanted from deep to shallow environments exhibited significant bleaching 8 weeks after transplantation but did not show any mortality after a year, whereas those transplanted from shallow to deep did not bleach, but 7 out of 37 colonies died. After surveying restriction-fragment-length polymorphisms (RFLP) in RNA genes, the surprising mortality results were explained by changes in the Symbiodiniaceae community. Corals only changed symbiont community to match their new depth when transplanted from deep to shallow sites, suggesting that bleaching was needed as a catalyst for symbiont switching. Without bleaching, unfavourable host-Symbiodiniaceae symbioses persisted under chronic stress, resulting in mortality. Baker does recognise, however, that this supposed strategy is extremely risky for the coral as it may result in starvation and mortality. Silverstein *et al.* (2015) also found that bleaching was required to change Symbiodiniaceae community, after experimentally bleaching the coral *Montastraea cavernosa,* which was initially dominated by *Cladocopium* (ITS2 type C3). Regardless of whether corals were bleached due to thermal stress or herbicide application, they took up previously undetected *Durusdinium* (ITS2 type D1a). These became the dominant symbiont and conferred thermal tolerance to the coral when thermal stress was applied for 10 days, three months after the initial experimental bleaching. Boulotte *et al.*

(2016) recently showed evidence for symbiont switching as well as shuffling in the pocilloporid species, *Stylophora pistillata* and *Pocillopora damnicornis*, following two consecutive bleaching events on the Great Barrier Reef. These corals were well known to associate with *Cladocopium* (formerly *Symbiodinium* Clade C) and it was generally accepted that their symbionts transmitted vertically from the 'mother' colony (Wicks *et al.*, 2010). Deep amplicon sequencing using the internal transcribed spacer 2 (ITS2) marker gene showed that most newly uptaken symbionts from the environment remained below 1% relative abundance in the holobiont. The most abundant new symbiont was a completely novel *Cladocopium* subgroup, and a further two belonged to the thermally resistant *Durusdinium* (Boulotte *et al.*, 2016). As alluded to previously, the rare and often overlooked symbionts may be important in providing mechanisms to survive environmental stress.

1.4.6. Symbiont shuffling

Symbiont shuffling has been the more frequently reported mode of Symbiodiniaceae community change. Berkelmans & van Oppen (2006) were the first to show, through transplantation and experimental manipulation, that thermal acclimatisation was causally linked to a shuffle in dominant symbiont type from *Cladocopium* to *Durusdinium* (Clade C to D) in *Acropora millepora*. Thermal tolerance of corals transplanted from the cooler southern Great Barrier Reef to the warmer central GBR increased in the range of 1-1.5°C after changing to *Durusdinium* dominance, while corals which did not shuffle from *Cladocopium* ITS2 type C2 dominance had the same lower thermal tolerance as native corals which had not experienced a warmer environment for 14 months. The authors suggested that while coral host defences such as HSPs and antioxidants can regulate the acclimation capacity of a coral to an extent, it was the Symbiodiniaceae hosted which ultimately determined the thermal tolerance of *Acropora millepora*.

Reciprocal transplantation in the field is an effective approach taken to investigate changes in symbiont composition. Steven Palumbi's research group use back reef pools in American Samoa with differing thermal regimes as natural experimental units. They have been able to study change in symbiont communities following transplantation between pools experiencing moderate and high fluctuation in temperature. Their results across several species showed that (contrary to Goulet, 2006) many corals hosted multiple Symbiodiniaceae genera and that the coral-algal partnerships often conformed to higher temperature environments by showing higher proportions of *Durusdinium* (Oliver & Palumbi, 2011b). However, their study of *A. hyacinthus* from these pools showed that despite hosting different Symbiodiniaceae communities, when subjected to heat stress, corals from more thermally stable pools bleached, regardless of their symbiont make-up, suggesting that symbiont clade did not play such a big role in thermotolerance (Oliver & Palumbi, 2011a). Sampayo *et al.* (2016) also adopted a translocation design to investigate coral symbioses but between depths at Heron Island, Australia. Although they showed evidence for uptake of local Symbiodiniaceae from the environment, new symbioses were not stable, reverting to phylogenetically constrained partnerships within a year.

1.4.7. Trade offs

Corals unable to change their Symbiodiniaceae communities to match their new local conditions paid the price of mortality under the additional stress of increased temperature (Sampayo *et al.*, 2016). Disproportionately high mortality of transplanted coral holobionts hosting foreign symbionts indicated that living outside their adaptive/acclimatory state came at a high energetic (and ultimate) cost as the host had to counterbalance its disadvantageous symbionts. It will be important to investigate whether any corals can rapidly adapt to new sub-optimal conditions by changing their Symbiodiniaceae communities.

Following experimental bleaching, Cunning *et al.* (2015) showed that symbiont shuffling toward heat-tolerant Symbiodiniaceae in *Orbicella faveolata* was greater when bleaching was severe and recovery occurred in a warmer environment than if bleaching was moderate and coral recovered in a cooler environment. However, there appeared to be a trade-off associated with hosting higher proportions of heat-tolerant Symbiodiniaceae; although bleaching resistance increased, photochemical efficiency decreased, suggesting that symbiont shuffling oppositely impacts stress tolerance and performance. The cost of hosting thermally tolerant (*Durusdinium*) symbionts has also been documented for juvenile *Acropora tenuis*, which grew at half the rate of those hosting *Cladocopium* at the same site (Little *et al.*, 2004). Further complicating matters, this trade-off can be affected by temperature. *Pocillopora damicornis* hosting heat-tolerant (*Durusdinium*) symbionts grew 40% slower than corals hosting *Cladocopium* at 26ºC, but this trade-off was eliminated with warming of 1.5-3ºC (Cunning *et al.*, 2015b). These results suggest that switching/shuffling to *Durusdinium* may be worth it after all, but only in hot conditions.

1.4.8. How can the role of zooxanthellae be disentangled from other factors?

As concluded by Baker (2001), changes in zooxanthellae community composition may be slow without the catalysis of a bleaching event to remove existing symbionts. Baker supposed that established symbionts had a competitive 'home-advantage' over incoming or low-abundance Symbiodiniaceae. The window of opportunity provided by bleaching events could allow unusual or low-abundance opportunistic symbionts, such as *Durusdinium* (Stat & Gates, 2011), which are better suited to stressful environmental conditions to colonise or increase in abundance. Rapid removal of symbionts can be done experimentally to investigate new symbiont relationships under controlled conditions. Chemical expulsion of algal symbionts has been done using copper (Jones, 2004), the herbicide DCMU (Jones, 2004; Silverstein *et al.*, 2015), and recently, menthol (Wang *et al.*, 2012).

1.5. Bacteria

The first studies of bacteria associated with coral focussed on their disease-causing potential, but it is now understood that coral-associated bacteria have wide-ranging roles in maintaining coral holobiont health and may hold the key to rapid holobiont adaptation.

1.5.1. Bacterial bleaching hypothesis

Eugene Rosenberg's research group at Tel Aviv University, Israel has been studying the role of bacteria in coral holobiont fitness for the last 20 years. They developed a controversial concept known as the 'bacterial bleaching hypothesis' (BBH) after proposing that annual bleaching of the Mediterranean/Red Sea coral *Oculina patagonica* was caused by the pathogenic bacterium *Vibrio shiloi* (Kushmaro *et al.*, 1996) and bleaching of *Pocillopora damicornis* was caused by *V. coralliilyticus* (Ben-Haim *et al.*, 2003). While this was hotly contested by Tracy Ainsworth and colleagues (2008), who were adamant that environmental stressors had caused the bleaching and bacteria were merely colonising opportunistically, the BBH and *O. patagonica* – *V. shiloi* model system did give way to another hypothesis.

1.5.2. Coral probiotic hypothesis

When Reshef *et al.* (2006) found that *V. shiloi* no longer caused bleaching in *O. patagonica*, they proposed that due to changes in the relative abundances of bacteria in the coral holobiont, the coral had adapted to new conditions, and coined this the 'coral probiotic hypothesis'. Change in bacterial community over seasons was previously recorded in *O. patagonica* (Koren & Rosenberg, 2006), and from this, Reshef *et al.*, (2006) surmised that environmental conditions could select for advantageous changes in bacterial community far faster than classical Darwinian gene mutation and selection in the coral host alone.

Considering that promotion of plant growth by manipulation of root-associated microbes and biological controls against plant pathogens are already implemented in farming (Dobbelaere *et al.*, 2003), and probiotic formulations are widely used in veterinary and human medicine, the use of beneficial microorganisms for corals (BMC) might not be so far-fetched in the search for potential solutions to the coral bleaching crisis (Krediet *et al.*, 2013; Peixoto *et al.*, 2017).

1.5.3. Role of bacteria in conferring heat tolerance

Ziegler *et al.* (2017) recently employed a reciprocal translocation experiment of *A. hyacinthus* between two thermally distinct back-reef pools to test the coral probiotic hypothesis. In contrast to the findings of Sampayo *et al.* (2016), after 17 months, the microbiomes of native and transplanted holobionts were indistinguishable, highlighting that coral holobionts are capable of forming new environment-specific symbioses. Interestingly, in short-term heating experiments, the corals which had spent the last 17 months in warmer, more variable pools bleached less and showed little shift in bacterial community. The thermally stable microbiomes were characterised by a persistent set of OTUs, mostly belonging to the *Alphaproteobacteria*, which were not hosted by the bleaching-susceptible corals. It remains to be seen whether these indicator associations between certain bacterial taxa and heat tolerant corals are due to the same heat-based selection pressures acting in parallel on both coral host, and bacteria, or whether differences in coral thermotolerance are caused by the microbial community hosted. A separate study showed bacteria to play a role in granting thermal tolerance to *P. damicornis* (Gilbert *et al.*, 2012). The removal of α and γ-*Proteobacteria* by antibiotics caused severe tissue loss during heat stress whereas corals with intact microbiomes only suffered typical heat-induced declines in photosynthetic efficiency.

Should a successful community of microbes provide stress tolerance to the coral, it would be advantageous for this microbiome to be inherited by future generations. However, vertical transmission has been rejected as a mode of bacterial transfer in *Pocillopora meandrina* (Apprill *et al.*, 2009) suggesting that uptake from the environment (horizontal transmission) may be important throughout a coral's lifespan. Sharp *et al.* (2010) were not able to detect bacteria in the eggs, sperm or larvae of seven mass-spawning corals, but were able to from the early settled stages of polyps, thereby also concluding that bacteria were not transferred vertically. Understanding the acquisition and transmission of coral-associated microbes are key areas yet to be fully understood. Since bacterial colonisation appeared to occur after settlement in several corals, there is huge potential for experiments to manipulate the bacterial assemblages of corals.

1.5.4. Antibiotic treatment

The use of antibiotics for the investigation of coral diseases is well established (Sweet *et al.*, 2014). However, the experimental manipulation of healthy corals with antibiotics to understand normal functioning is a new approach. The positive roles bacteria play to maintain coral health and their potential to increase resilience of corals to environmental stress warrant continued experimentation (Mouchka *et al.*, 2010). Bacterial community shifts have been recreated in the laboratory with the use of antibiotics and bacterial uptake from the corals' native environment investigated (Sweet *et al.*, 2011b). What remains to be tested is whether, like the adaptive bleaching hypothesis, uptake of locally adapted bacteria can be accelerated by removal of poorly adapted symbionts to promote corals better adapted to their surroundings. Antibiotic administration was inadvertently used to explore the *Daphnia* holobiont (Gorokhova *et al.*, 2015). When antibiotics were administered to simulate pollution in freshwater systems, it was found that due to changes in their gut microbiota, the water fleas reduced feeding. Similar links between microbiome structure and behaviour have been

observed in germ-free mice and rats (reviewed by Moloney *et al.*, 2014). This highlights just how complex the relationship between host and microbiome can be.

To conclusively test whether differential coral bleaching responses are owed to their microbiomes, experimental manipulation is needed. Ziegler *et al.* (2017) suggested transferring purportedly heat-resistant symbionts from corals residing in high temperature variation pools to corals from thermally stable pools to determine whether corals could acquire heat tolerance. Acquisition of heat tolerance was recently demonstrated in aphids by replacing a single obligate bacterial strain (Moran & Yun, 2015), but these methods are yet to be developed in coral biology. The implementation of such active intervention in areas we do not yet fully understand have raised logistical, ecological and ethical concerns (Sweet *et al.*, 2017a).

1.6. Archaea

Archaea are famously known for their existence in extreme environments, though we now know these 'extremophiles' to be widespread (DeLong, 1998). Archaea have been found to comprise nearly half the prokaryotic community, at > 10^7 cells cm^{-2}, in the *Porites astreoides* holobiont (Wegley *et al.*, 2004). Due to their propensity to thrive in extreme habitats, with respect to temperature, pH, salinity, and anoxia, archaea may be even better than coral-associated bacteria at continuing to function in the face of change. Archaea are renowned for their unusual modes of garnering energy, using organic compounds i.e. sugars, inorganic compounds e.g. ammonia and sulphur (lithotrophs), sunlight (phototrophs; different to autotrophs as archaea do not perform photosynthesis) to even metal ions or hydrogen (Rosenberg *et al.*, 2014). Their unusual properties and modes of metabolism may allow archaea to provide functions to the coral holobiont which other taxa cannot. The majority of research on corals so far points toward archaeal roles in nitrogen cycling (Siboni *et al*., 2008; Rädecker *et al.*, 2015), although much remains unknown. Unlike the algal and bacterial

symbionts, archaea do not seem to form species-specific associations with coral hosts (Wegley *et al.*, 2004), rather, location appears to play a greater role in their community composition (Siboni *et al.*, 2012), suggesting they may be more easily swapped to suit local conditions.

1.7. Fungi

The presence of coral-associated fungi has been known for over 40 years (Kendrick *et al.*, 1982), and, as was common for early microbiological studies, was linked to disease symptoms (Geiser *et al*., 1998). Most famously, the aspergillosis disease of sea fans was purportedly caused by the fungi *Aspergillus sydowii* (Geiser *et al.*, 1998), though more recent evidence suggests that more than one opportunistic fungal species may be implicated in aspergillosis and related sea fan diseases (Barrero-Canosa *et al.*, 2013). Early studies of Ascomycetes-like fungi in the massive coral, *Porites lobata,* noted that fungal associations took place early in a coral's life to grow with the coral skeleton just beneath the tissue (Le Campion Alsumard *et al.*, 1995). More recent meta-barcoding of 31 coral skeletons spanning 12 coral genera revealed the most abundant and prevalent fungi belonged to the *Lulworthia* and *Lulwoana* (Ascomycota), which are both known saprotrophs (meaning they feed on decaying organic matter; Góes-Neto *et al.*, 2020). There are relatively few studies which detail the roles of fungi in healthy corals, though amplicon sequencing of the small subunit rRNA gene and transcriptomic analysis of the fungal community associated with the coral *Acropora hyacinthus* revealed a diverse, metabolically active community (Amend *et al.*, 2012). The same study also revealed a core assemblage of fungi correlated more strongly with the host than with environmental conditions or Symbiodiniaceae identity, suggesting a host-specific partnership (Amend *et al.*, 2012). There were some specific fungal OTUs associated with corals living in warm pools, but aside from a few core taxa, most fungal OTUs could either be classed as transient or found only in specific locations, similar to patterns observed in coral-associated bacteria (Amend *et al.*, 2012; Hernandez-Agreda *et*

al., 2016b). In-depth meta-barcoding of the fungal 18S rRNA gene revealed that almost 8% of sequences could not be assigned to any known fungal lineage, highlighting the scarce knowledge of coral-associated fungi (Góes-Neto *et al.*, 2020). Meta-genomic analyses suggest that endolithic fungi could be responsible for nitrogen cycling (ammonia assimilation) within the coral holobiont (Wegley *et al.*, 2007). Similar to opportunistic bacteria which convert from being commensal to pathogenic under certain conditions, coral-associated fungi may form different relationships with their host based on environmental conditions (Le Campion Alsumard *et al.*, 1995), therefore it remains uncertain as to whether fungi can benefit corals facing environmental stress.

1.9. Viruses

A recent and rapidly growing area of coral biology focuses on identifying viruses and their potential functions in the coral holobiont. The advent of metagenomic techniques has permitted documentation of a high diversity of DNA and RNA viruses (Weynberg *et al.*, 2014). While little is known about their specific functions, it is likely that they are important in structuring the coral's prokaryotic community (Bourne *et al.*, 2016). Phages are viruses which infect bacteria and are thus found wherever bacteria exist (Wegley *et al.*, 2007). To replicate, they inject their genome into the cytoplasm of bacteria (Vega Thurber *et al.*, 2017). Phage therapy to treat the coral pathogen *Vibrio coralliilyticus* has been experimentally trialled on pure cultures of *Symbiodinium* and coral juveniles; the Myoviridae bacteriophage known as YC was able to prevent *V. coralliilyticus*-induced photoinactivation and tissue lysis (Cohen *et al.*, 2013). This highlights another mode in which the coral hologenome can rapidly change, and could be harnessed for microbiome engineering (Epstein *et al.*, 2019).

1.10. Natural laboratories

Marginal habitats with naturally extreme environmental conditions can be used as so-called 'natural laboratories' so that researchers can learn from existing stress-tolerant coral populations and their holobiont compositions (Camp *et al.*, 2019). Marginal habitats can be defined as environments where corals live close to their environmental limits (Kleypas *et al.*, 1999; Perry & Larcombe, 2003). Therefore, different marginal habitats can be sub-optimal or stressful for different reasons. For example, mesophotic reefs and turbid nearshore environments host lower than optimal light conditions, high-latitude reefs host cooler temperatures, CO_2 vent sites have lower pH and aragonite saturations, while intertidal and nearshore habitats, including seagrass beds and mangroves, host a raft of fluctuating and multiple stressors such as extreme temperature, pH, and dissolved oxygen (Camp *et al.*, 2017, 2018). Resilient coral populations and their consortium of associated microorganisms hold potential for utilisation in understanding the mechanisms behind coral stress-tolerance and resilience, as well as scope for active conservation measures. Corals living in marginal habitats are only now being recognised for their conservation potential given the imminent threats posed to typical reefs (Rivest *et al.*, 2017). These resilient corals might have the potential, as genetic stock, to re-seed reefs following declines. The phenotypic traits of these already stress-resilient corals could also be artificially selected for in selective breeding programmes which apply specific stressors, much faster than adaptation to naturally changing conditions on the reef (known as assisted evolution; van Oppen *et al.*, 2015). Similar techniques involving selection of the coral's symbionts have been coined under the umbrella term 'microbiome engineering': defined as the experimental manipulation of individual microorganisms and microbial communities (Epstein *et al.*, 2019). Experimental evolution of Symbiodiniaceae has already shown potential for rapid adaptation to higher temperatures, with algae selectively cultured over a year (equating to 41-69 asexual generations) demonstrating faster growth rates and higher photosynthetic efficiencies under

acute heat stress, than wild-type populations (Chakravarti & van Oppen, 2018). Rapid adaptation of coral holobionts by microbiome engineering might also be achieved by inoculation of the coral host with beneficial microorganisms. Such prospective development of coral probiotics from beneficial microorganisms for corals (BMCs) is currently underway (Peixoto *et al.*, 2017); with putatively beneficial native bacteria isolated from the coral *Pocillopora damicornis* and surrounding seawater showing promise against a pathogen challenge treatment with *Vibrio coralliilyticus* (Rosado *et al.*, 2019). Known naturally stress-resistant or resilient corals living in marginal habitats might represent a good starting point to search for further putatively beneficial microorganisms for corals.

1.11. Conclusion

The mechanisms which permit corals to survive under extreme conditions are diverse, and reliant not only on the coral host, but often on a suite of microbial symbionts. While the coral host provides a huge source of genetic diversity on which environmental selection can act upon (comparable or larger than the human genome; ReFuGe 2020 consortium), adaptation may also depend on the genes of all of a coral's symbionts (*sensu* Hologenome Theory; Zilber-Rosenberg & Rosenberg, 2008). And while rapid acclimatisation to changing conditions is governed by the phenotypic plasticity and history of the coral host, intermediate options such as symbiont switching or shuffling are also available to varying extents. Resistance to bleaching is almost certainly provided as a culmination of physiological and biochemical traits from the whole holobiont, including host, algal symbionts, and assemblage of other symbiotic microorganisms.

The coral host and symbiotic algae have been studied extensively in terms of their thermotolerance and contribution to the adaptive capacity of coral holobionts facing future global change. The remaining microbial partners of the holobiont are now receiving increased attention, but substantial knowledge gaps remain. Advances in sequencing

technology continue to provide insight into the complex relationships between host, Symbiodiniaceae, and the remaining microbiome, including their co-evolution, collective functions, molecular mechanisms behind maintenance of the holobiont, and the role of the microbiome in holobiont acclimatisation/adaptation to environmental change (Bourne *et al.*, 2016; Sea-quence project, ReFuGe 2020, Voolstra *et al*., 2015).

The main aim of this project was to establish the contribution of microbial symbionts to local adaptation of the holobiont by testing the coral probiotic hypothesis (Reshef *et al.*, 2006). This thesis addresses some of the yet unanswered questions on the potential of marginal habitats to 'pre-adapt' coral holobionts to future environmental change with a view that findings may be able to influence active conservation.

1.12. Synopsis

Corals, like all animals, can match their physiology to the local environment through either phenotypic plasticity (acclimatisation at the individual colony level; Oliver & Palumbi, 2011a) or adaptation (changes in the gene pool caused by natural selection; Kenkel *et al.*, 2013a). Corals, as meta-organisms, known as holobionts, also have the ability to respond to their environment by changing the composition of their symbiotic community (Berkelmans & van Oppen, 2006). This can be considered a rapid intermediate response mechanism: a potentially reversible (i.e. plastic) change in genotype frequencies. Previous studies have shown that corals living in different thermal environments, whether this be differences in mean temperature, maximum temperature, temperature range, or frequency in temperature fluctuation—even across small spatial scales—can exhibit significant differences in thermal tolerance (Oliver & Palumbi, 2011a). Furthermore, acute temperature pulses have been shown to induce increased thermal tolerance (Middlebrook *et al.*, 2008).

In order to firstly understand the physiology, in particular the thermotolerance, of conspecific corals from habitats with different temperature regimes, an *ex situ* common-garden experiment was conducted, with increasing temperature as a stressor (Chapter 2). The reef-building coral, *Porites lutea*, was subjected to 20 days of heat-ramping, reaching temperatures above the local average summer maximum, to simulate the prolonged temperature exposures of marine heatwaves. Sustained elevated temperatures were used to test thermotolerance limits as opposed to a short, sharp heat-shock since bleaching is usually the result of accumulated stress over an extended period of time (Fig. 1.2).

Survival during extreme temperature conditions is dependent not only on the coral host, but on the capacity of a coral holobiont as a whole, including symbiotic microorganisms. The coral holobiont comprises a cnidarian host, endosymbiotic algae, and a diverse array of bacteria, fungi, archaea and viruses (Rohwer *et al.*, 2002). While it has become apparent

that both host and symbiotic microorganisms are involved in determining holobiont thermotolerance limits (Oliver & Palumbi, 2011b; Ziegler *et al.*, 2017), the relative contributions of each partner are difficult to partition. Genotyping both hosts and microbial symbionts from conspecific coral holobionts existing under different thermal regimes can help to disentangle which partner in the holobiont may be driving an adaptive response.

For this reason, a reciprocal translocation experiment of conspecific corals from mangrove and fore-reef habitat was conducted, and DNA samples were collected. Translocation of conspecific corals between habitats was performed to test for local adaptation of the coral holobionts and to test whether horizontal transmission of microorganisms from the environment to the coral holobiont would occur (Chapter 3). Further manipulation of the coral microbiome by antibiotic administration was undertaken to initiate/accelerate re-shuffling of the microbiome, which culminated in a fully factorial antibiotic treatment × reciprocal translocation experiment (Chapter 4).

1.12.1. Study sites

The research contained in this thesis centres around two key bioregions on either side of the Indian Ocean. Sites were chosen in order to test hypotheses and compare patterns between reef and marginal environments, across geographic locations. This approach is often taken to study convergent adaptive evolution, whereby environmental selection drives adaptation of the same trait, independent of geographic location. For example, mangrove trees themselves, despite not belonging to one phylogenetic clade, or originating from one geographic location, have all convergently evolved to tolerate saltwater immersion (Lyu *et al.*, 2018). Site selection allowed comparison of coral species found in marginal mangrove habitats on either side of an ocean basin, as well as identification of coral-associated microbial communities which are common to mangrove environments, irrespective of geography.

Curieuse Marine National Park, Seychelles, Western Indian Ocean

The Western Indian Ocean (WIO) is home to 16% of the world's coral reefs and is thought to be the second most biodiverse coral region, after the Coral Triangle (Obura, 2012; Obura *et al.*, 2017). It is also a region impacted by a myriad of threats to coral survival, including an extreme thermal history, coupled with anthropogenic threats from over-fishing and coastal development. The WIO was one of the regions hardest hit by the 1998 global mass-bleaching event, with catastrophic coral cover losses averaging 25% (Wilkinson *et al.*, 1999; Goreau *et al.*, 2000), and has since suffered further bleaching episodes in 2005, 2010, and most recently 2016 (Obura *et al.*, 2017).

The Seychelles in particular suffered some of the greatest coral mortality following the 1998 El Niño with many sites reduced to only 5% coral cover (Turner *et al.*, 2000; Graham *et al.*, 2008). After almost two decades of promising coral reef recovery, the Seychelles was again one of the worst hit countries, this time by the 2016 Godzilla El Niño, with extreme bleaching (> 50% coral cover bleached) reported for over half of reef sites (Obura *et al.*, 2017). Such devastation to the reefs of the Seychelles are particularly concerning given the dependence of the Seychelles' economy on fisheries and tourism.

This project began following the 2016 mass-bleaching, so the first expedition to the Seychelles for this project was somewhat of a reconnaissance mission to determine which coral species, within which sites, had persisted.

Both fore-reef and mangrove sites in the Western Indian Ocean were located within Curieuse Marine National Park (CMNP), Seychelles (Fig. 1.6 B). The fore-reef site (Home Reef; Fig. 1.7 A) was situated adjacent to the fringing reef crest (4° 17' 05.1" S, 55° 44' 07.6" E), between the bays known locally as Baie La Raie and Anse Papaie off the south coast of Curieuse Island. The mangrove site (Turtle Pond; Fig 1.7 B) was situated behind a fallen sea wall within Baie La Raie (4° 17 '12.9" S, 55° 43' 49.1" E). The sea wall

was originally built in 1910 to enclose a 40-acre pond for raising hawksbill turtles. The turtle nursery project was unsuccessful, but the sheltered environment allowed mangrove trees (including *Avicennia marina, Rhizophora mucronata*, *Lumnitzera racemosa*, and *Bruguiera gymnorrhiza*) to proliferate (Beasley *et al.*, 2018). In 2004, the 'Boxing Day Tsunami' knocked over part of the sea wall, providing hard substrate for coral to settle and grow on, and a sheltered nursery for lemon sharks (Obura & Abdulla, 2005).

Wakatobi Marine National Park, Indonesia, Central Indo-Pacific Ocean

The Central Indo-Pacific (CIP) is a hotspot of coral diversity (Hughes *et al.*, 2002). There are 627 scleractinian coral species described from The Coral Triangle, which accounts for 74% of all coral species worldwide (Veron *et al.*, 2015). In comparison with the Western Indian Ocean, the Central Indo-Pacific has seemingly suffered fewer mass-bleaching events with the '50 reefs' initiative reporting several 'bioclimatic units' with promising thermal histories (Beyer *et al.*, 2018) and 30% of reefs with stress-moderating turbidity situated in the Coral Triangle (Sully & van Woesik, 2020).

Reef and mangrove coral habitats for the Indo-Pacific were located within the Wakatobi Marine National Park, Southeast Sulawesi, Indonesia (Fig. 1.6 C). In comparison with the reef habitat studied in the Seychelles, the reefs of the Wakatobi had not suffered such recent rapid declines in coral cover, but rather a steady decline from 45% cover (with a range of 40-70%) in 2002 to 20% in 2011, remaining stable at 19.5% in 2014 (Marlow *et al.*, 2019).

The fore-reef habitat was situated off the southwest coast of Hoga Island, adjacent to the fringing reef crest, at a site known locally as 'Buoy 2' (5° 28' 31.2" S, 123° 45' 32.0" E). While there is some evidence for bleaching-related declines at this site (consistent with degree heating months recorded in 2002, 2005 and 2006; Gouraguine *et al.*, 2019), Buoy 2 had also previously been impacted by other human stressors, including the construction of a jetty (Caras & Pasternak, 2009), and possibly bomb-fishing (Crabbe *et al.*, 2004), the culmination

of which have resulted in increases in abiotic cover such as rubble (Gouraguine *et al.*, 2019). The marginal habitat, a thermally-variable tidally-influenced lagoon, was within a mangrove system characterised by *Rhizophora stylosa* trees, located at the northern coast of Kaledupa Island and known locally as ‘Langira’ (5° 28' 41.1" S, 123° 43' 17.4" E). Mangrove forests in the Wakatobi National Marine Park are unusual as they have formed atop shallow calcium carbonate-rich sediment and fossil coral which hampers root development, rather than deep, fine-grained sediment (Cragg & Hendy, 2010). Subterranean channels caused by the dissolution of coral rock by low-pH mangrove sediment also allows circulation of seawater belowground (Cragg & Hendy, 2010).

The coral reefs and marginal coral habitats studied in this thesis are supposed to be broadly representative of declining reefs and neighbouring mangrove ecosystems worldwide. While scientists cannot perfectly simulate the effects of a changing climate on scleractinian corals, these marginal coral habitats provide an imperfect parallel to study coral resilience and microbiome composition in response to environmental extremes.

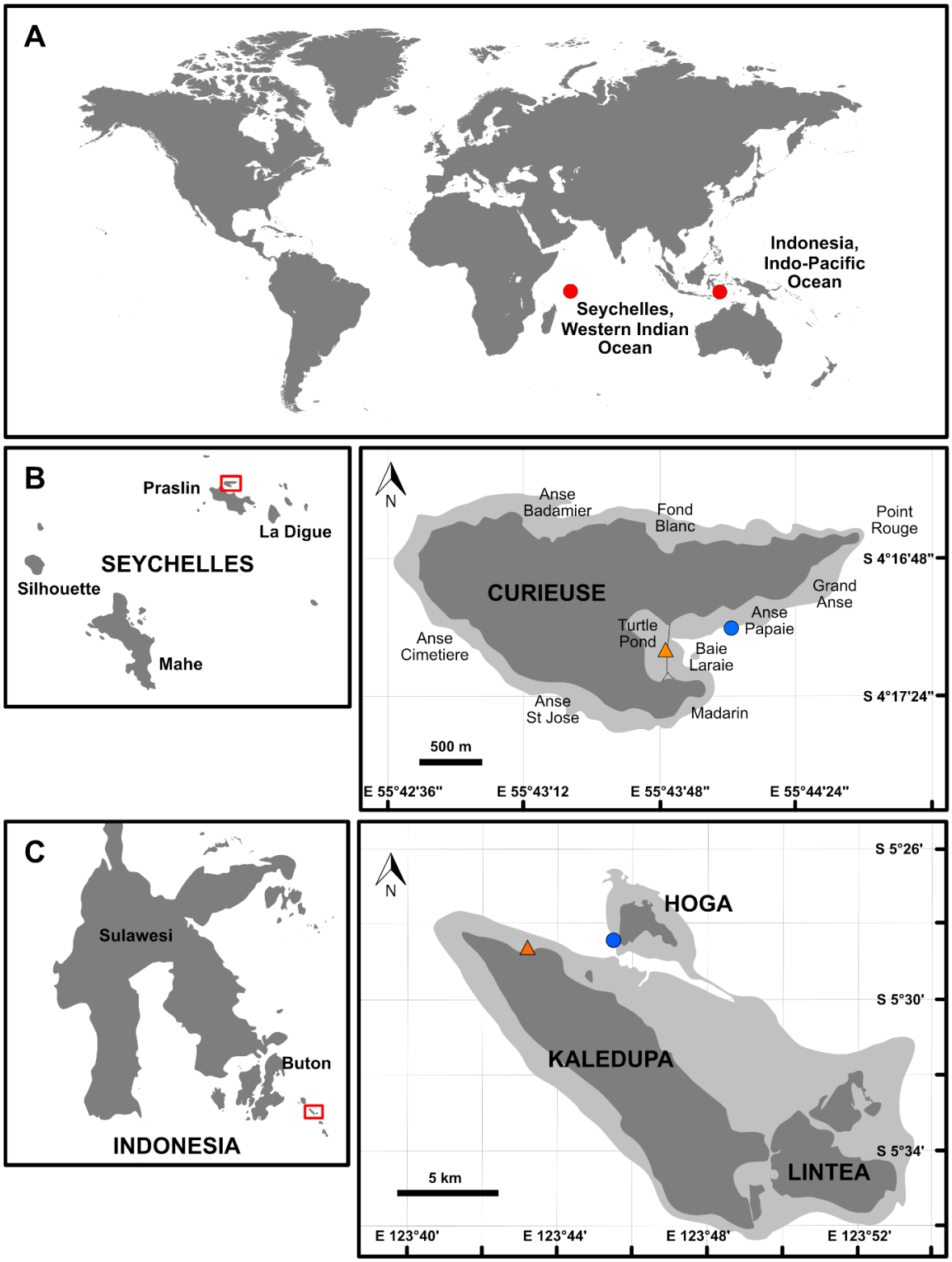

Figure 1.6. A) Study sites in the Seychelles, Western Indian Ocean, and Indonesia, Indo-Pacific Ocean. **B)** Curieuse island, Curieuse Marine National Park (CMNP), Seychelles shown in a red box. Fore-reef site (Home Reef: blue circle) between Baie La Raie and Anse Papaie, and mangrove site (Turtle Pond: orange triangle) within Baie La Raie. **C)** Hoga and Kaledupa islands, Wakatobi Marine National Park (WMNP), Indonesia (red box). Reef site (Buoy 2: blue circle) off southwest coast of Hoga island, and mangrove site (Langira mangrove: orange triangle) off northern coast of Kaledupa island.

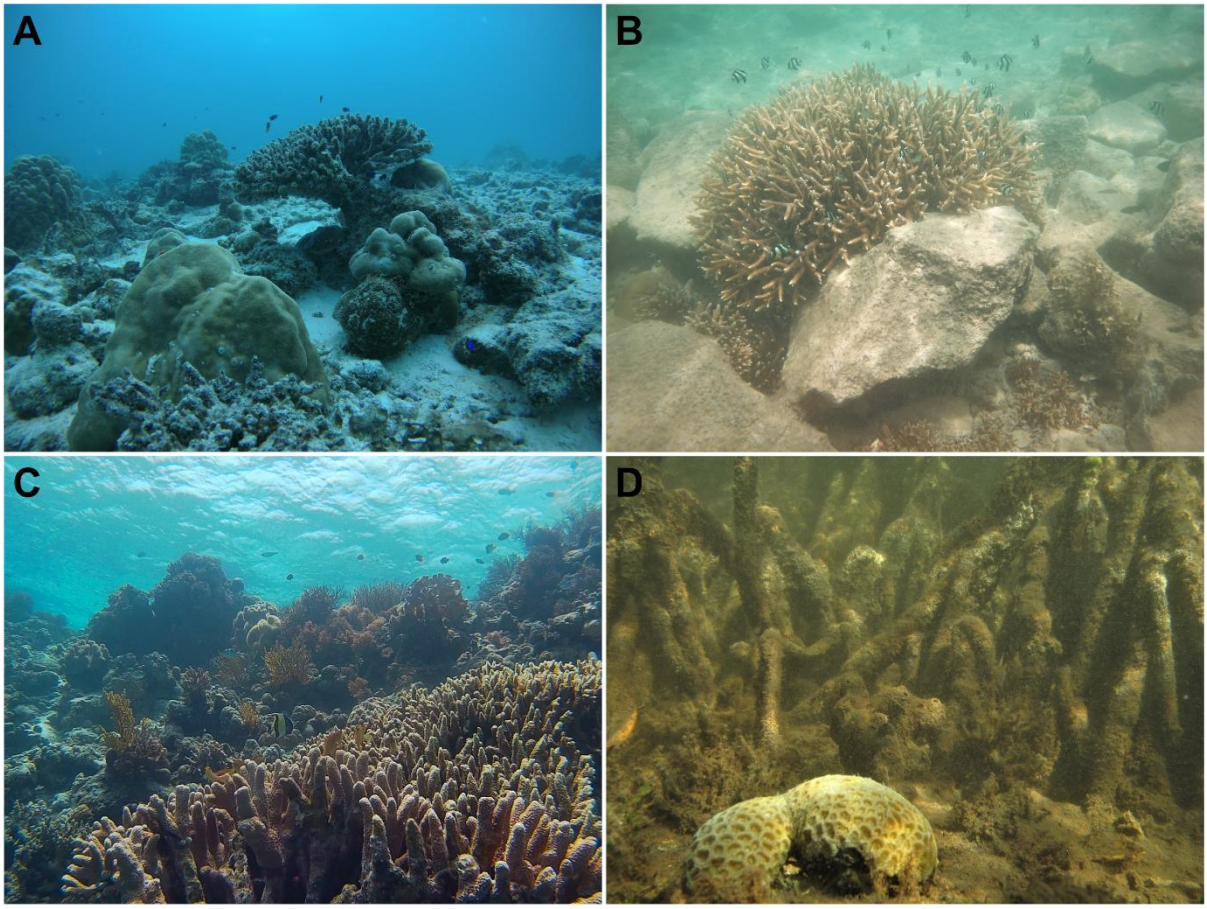

Figure 1.7. Photographs of contrasting reef and mangrove habitats in the Seychelles and Indonesia, taken in 2017. **A)** Dead *Acropora* and live *Porites lutea* colonies at Home Reef, Seychelles, following the 2016 El Niño. **B)** Live colony of *Acropora muricata* in Turtle Pond mangrove, Seychelles. **C)** Buoy 2 fore-reef dominated by branching *Porites* species in Indonesia. **D)** A pale colony of *Dipsastraea* cf. *pallida* living in Langira mangrove, Indonesia.

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Chapter 2: Variable temperature mangrove habitat offers modest pre-conditioning to climate risk hard coral, *Porites lutea*

Abstract

Coral reefs worldwide are declining due to mass bleaching events caused by marine heatwaves. Emerging evidence from naturally extreme environments has provided insight into how corals survive extreme temperatures, however, little is known about the thermal physiology and tolerance limits of mangrove-dwelling corals. This study compared the thermal tolerance limits of the reef-building coral, *Porites lutea*, from a marginal mangrove habitat and from a neighbouring fore-reef. Langira mangrove experiences temperatures as high as 37.7°C and daily fluctuations of up to 7°C, compared with Buoy 2 fore-reef with a maximum temperature of 31.4°C and daily range of up to 3°C. Corals from both habitats were subject to *ex situ* experimental thermal stress, up to 36°C, over 20 days. Productivity (P) and respiration (R) were measured as proxies of coral holobiont fitness, while bleaching status was assessed by algal symbiont density and chlorophyll *a* content. Corals from habitats of differing thermal regimes, mangrove and fore-reef, showed no difference in their metabolic response to heat stress with P/R ratios decreasing from > 1.5 to < 1, regardless of habitat. Mangrove corals hosted, on average, ≥ 45% higher algal symbiont densities and ≥ 37% higher chlorophyll *a* concentrations than reef corals throughout the experiment, suggesting different, habitat-driven, physiological strategies. Following 15 days of experimental heating, average symbiont density of reef corals was 53% lower than controls, while heated mangrove corals hosted only 32% lower average symbiont density than

controls. The more severe bleaching observed in heat-stressed reef corals, relative to mangrove corals, was also the result of a down-regulation in chlorophyll *a* concentration per symbiont cell (38% difference between heated and control reef corals, vs. only 1% difference observed in mangrove corals). So, in contrast to previous studies, variable temperature habitats may only offer slight pre-conditioning to corals facing future ocean warming.

2.1. Introduction

Worldwide, coral reefs are in crisis due to more frequent and severe mass bleaching events, caused by prolonged periods of elevated sea surface temperatures, against a background of global warming and ocean acidification (Hughes *et al.*, 2003; Bellwood *et al.*, 2004; Hughes *et al.*, 2017). However, there are coral communities which persist in naturally extreme or 'marginal' habitats, some of which are already experiencing the conditions predicted for reefs across the next 100 years of climate change (Camp *et al.*, 2018). Corals living in these habitats and surviving beyond the previously-defined environmental thresholds for coral existence provide some optimism for coral survivability under future climate scenarios. Marginal habitats are environments where coral communities live close to their environmental tolerance limits, in sub-optimal, or fluctuating physicochemical conditions (Kleypas *et al.*, 1999; Perry & Larcombe, 2003). Such sub-optimal conditions for coral survival include the low light of turbid and mesophotic reefs; cool temperatures of reefs at high latitudes or near upwellings; low pH and aragonite saturation of CO_2 vent sites; and the fluctuating and multiple stressors (including extreme temperatures) of intertidal, seagrass, and mangrove habitats (Camp *et al.*, 2018).

Avoiding mass bleaching and mortality amidst marine heat waves is arguably the biggest challenge currently facing reef-building corals (Hughes *et al.*, 2017). Corals from thermally variable habitats have been shown to have higher thermal bleaching resistance than their conspecifics found in moderate-temperature habitats (Oliver & Palumbi, 2011; Palumbi *et al.*,

2014). This is supported by further evidence for environmentally-mediated bleaching resilience, as corals in certain environments continue to survive beyond their regional bleaching threshold (Oliver & Palumbi, 2011; Riegl *et al.*, 2011). The back-reef pools of Ofu Island, American Samoa have become a model system for testing the acclimatisation effects of variable-temperature habitats on corals (Oliver & Palumbi, 2011; Palumbi *et al.*, 2014; Thomas *et al.*, 2018). The extreme temperature variation exhibited in the most variable pool ranges from 24.5 to 35°C, and fluctuations of 6°C occur daily (Thomas *et al.,* 2018). The effects of similarly extreme temperature fluctuations (ranging 7°C daily, up to a maximum of 37°C), as well as tidal exposure, have also been explored for corals inhabiting the intertidal reef flats of the Kimberley region in northwest Australia (Schoepf *et al.*, 2015).

Temperature fluctuations recorded in mangrove-influenced coral habitats are just as extreme (e.g. > 7°C range in Woody Isles mangrove lagoon on the Great Barrier Reef; Camp *et al.,* 2019), and accompanied by a suite of other stressors to coral, including low pH and oxygen levels (Camp *et al.*, 2018), terrestrial nutrient influx, freshwater inundation, aerial exposure, and biotic factors such as algal competition and predation (Yates *et al.,* 2014). Yet few marginal mangrove coral habitats have been characterised so far; namely Hurricane Hole off St. John Island of the US Virgin Islands *(Yates et al.*, 2014; Rogers, 2017), Turtle Pond of Curieuse Island in the Seychelles (Camp *et al.*, 2016b), Langira mangrove system off Kaledupa Island in the Wakatobi, Indonesia (Camp *et al.*, 2016b), Bouraké mangrove lagoon, New Caledonia (Camp *et al.*, 2017), and Woody Island and Howick Island within the Great Barrier Reef system, Australia (Camp *et al.*, 2019).

Variable-temperature habitats offer useful systems to test the time scales and levels to which corals may acclimatise or adapt to future warming seas. Thermal history can modify the thermal threshold of reef-building corals (Middlebrook *et al.*, 2008). Corals living at the edges of their physiological niches, in these marginal habitats, are expected to be acclimatised and possibly adapted to extreme conditions (Palumbi *et al.*, 2014). Therefore, mangroves, as

marginal coral habitats, are becoming ever more appealing as natural laboratories to test the adaptive capacity of reef-building coral, and as windows into the future structure and function of coral reefs. However, to date, little regarding the thermal physiology of mangrove-dwelling corals has been experimentally tested even though this is an essential first step to developing more complex hypotheses on the adaptive capacity of corals from extreme environments. Testing the thermal limits of these corals is paramount before declaring marginal habitats as key to the fight against coral mass extinction.

This study tested for local thermal acclimatisation and/or adaptation in *Porites lutea* from two thermally distinct habitats in the Wakatobi Marine National Park (WMNP), Indonesia. Despite their close proximity (< 3 km), corals in the mangroves experience greater diurnal temperature fluctuations than those from the thermally stable fore-reef, due to tidal influence on a shallow-water environment, and reduced water velocity and exchange. We hypothesised that since mangrove corals experience extreme temperature changes on a daily basis, they are better equipped to survive heat stress than conspecifics from a more thermally stable environment. To test these hypotheses, a laboratory-based common-garden experiment was set up, and increasing heat stress applied, to compare responses of *Porites lutea* from two different thermal environments. This species was selected as the study organism as it is a cosmopolitan reef-building coral and is the dominant massive coral species in both fore-reef and mangrove habitats (Veron, 2000; Camp *et al.*, 2016a). Responses measured included productivity and respiration, algal symbiont density, and chlorophyll *a* concentration. Conspecific corals were subjected to temperatures in excess of the Wakatobi thermal bleaching threshold (1°C above the local average summer maximum of 31°C, as defined by NOAA Coral Reef Watch) to establish coral thermal tolerance limits.

2.2. Methods

2.2.1. Habitat characterisation

Coral collection sites were located within the Wakatobi Marine National Park, Southeast Sulawesi, Indonesia (Fig. 2.1). The fore-reef site was situated off the south coast of Hoga Island, adjacent to the fringing reef crest, at a site known locally as ‘Buoy 2’ (5° 28' 31.6'' S, 123° 45' 32.5'' E). The mangrove site was characterised by *Rhizophora stylosa* trees, located at the northern coast of Kaledupa Island and known locally as ‘Langira’ (5° 28' 41.1'' S 123° 43' 17.4'' E). To characterise the environmental conditions of each site, temperature and light were recorded using HOBO Pendant® Temperature/Light 64K Data Loggers (Model UA-002-64, ONSET, USA) (Fig. 2.1 inset).

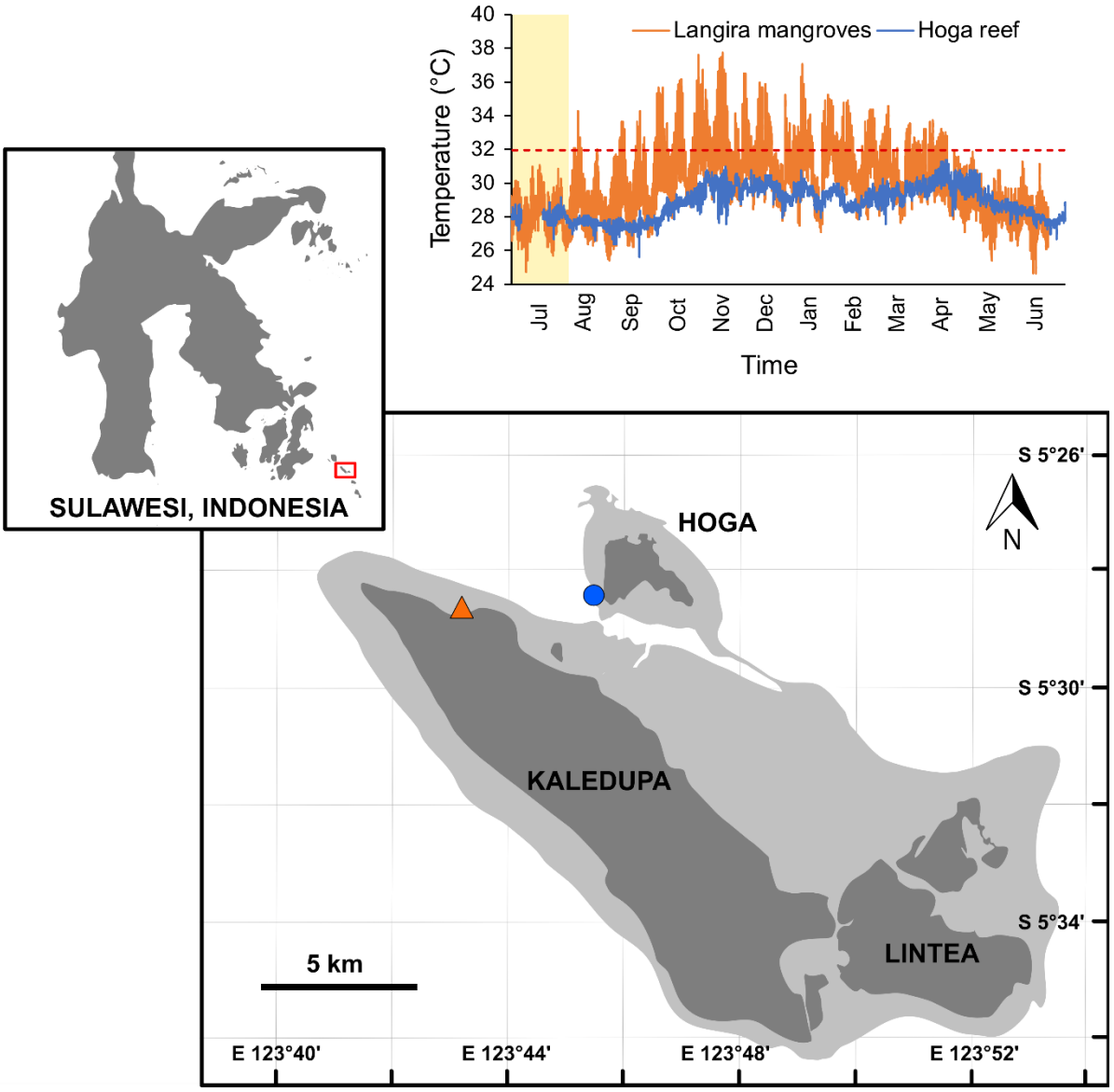

Figure 2.1. Coral collection sites within the Wakatobi Marine National Park. Hoga fore-reef marked on the map by a blue circle and Langira mangrove as an orange triangle. Top-left inset shows position of Kaledupa in southeast Sulawesi, within a red box. Top-right inset shows time series of sea temperature for Hoga fore-reef (blue) and Langira mangrove (orange) from July 2017 – July 2018. Data collected by HOBO loggers. Dashed red line indicates the Wakatobi regional bleaching threshold of 32°C = 1°C above the mean summer maximum. Yellow shaded area demarcates the period when the heating experiment was conducted.

2.2.3. Coral collection

On 12th July 2017, eight colonies of *Porites lutea* were taken from both fore-reef and marginal mangrove environments, ensuring at least 5 m between colonies to reduce the

likelihood of sampling asexual clones (as per Barshis *et al.,* 2010). After collection, colonies were immediately returned to Hoga Island Research Station and fragmented into four.

2.2.4. Tank environment

Eight 14.5L transparent polypropylene tanks were filled with 12L of seawater and connected to a flow-through system from the adjacent home reef. Therefore, corals were provided with natural particulate and dissolved organic matter as well as dissolved inorganic nutrients by the incoming seawater (as in Schoepf *et al.*, 2015). The water inflow rate was 300 ml/min, and effective water exchange (time taken until 99% of water in a tank is new) was T_{99} = 184 minutes. Each tank was equipped with a 200 W EHEIMthermocontrol aquarium heater and StreamON 3000 pump (EHEIM, Germany).

Coral fragments were kept shaded under the same light intensity to avoid high light stress (Kenkel *et al.*, 2013), and allowed to acclimate at 28°C (the local average SST for that time of year; Fig. 2.1 inset) for 5 days. After acclimation, one fragment per colony was randomly assigned to control treatment, and another fragment from the same colony assigned to 'heated' temperature treatment. There were two tanks per temperature treatment, per native habitat, resulting in four fragments per tank (n = 8 per habitat, per thermal regime). Tanks were cleaned twice weekly and salinity remained constant at 35 ppt throughout the experimental period.

2.2.5. Temperature treatment

Control tanks were maintained at 28˚C while heat treatment tanks were increased by 1˚C d^{-1} and held at 30, 32, 34, and 36˚C consecutively for 3 days. A HOBO temperature logger in each tank recorded seawater temperature every 15 minutes. The mean temperature of control aquaria across the duration of the experiment was 28.29°C (range: 26.49 – 29.65

°C). Temperature ramping of heated aquaria began on day 4, resulting in an overall heating rate of 0.48 °C d^{-1} (regression from day 4; R^2 = 0.94; range: 26.33 – 36.03 °C; Fig. 2.2).

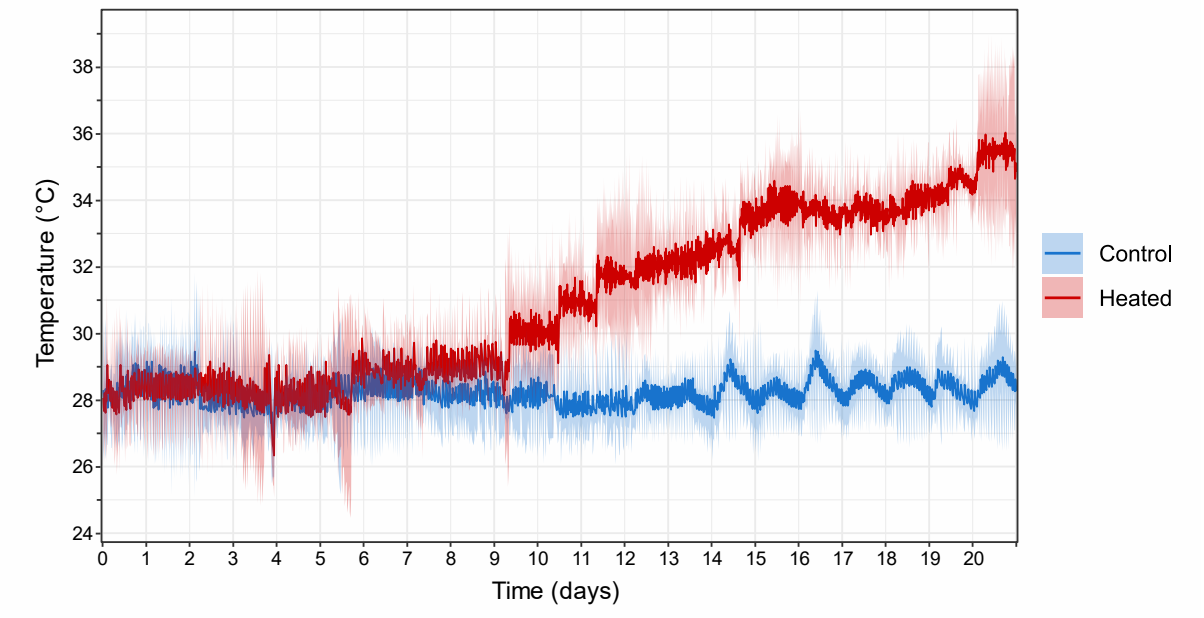

Figure 2.2. Temperature regimes during the 20-day experiment of control (blue) versus heated (red) aquaria (mean ± SE; *n* = 4). Control aquaria were set to 28°C throughout the experiment. Temperature ramping of heated aquaria began on day 5.

2.2.6. Sample collection time-points

Sacrificial fragments were sampled for chlorophyll quantification and symbiont density immediately after coral colony collection (native; *n* = 8 per habitat), and following 5 days of aquaria acclimation (*n* = 8 per habitat; Fig. 2.3). Final control and heated samples were taken at the end of the experiment on day 18 from coral fragments subjected to each experimental temperature regime (*n* = 8 per habitat, per thermal regime; Fig. 2.3). The experiment was planned to end before coral fragments died to avoid sampling tissue exhibiting necrosis. Upon the first signs of mortality in heat treatments, tissue samples were taken at 34˚C. However, to establish hard upper thermal limits, temperature ramping was continued to 36˚C, where despite whole fragment bleaching, the coral still provided a metabolic signature.

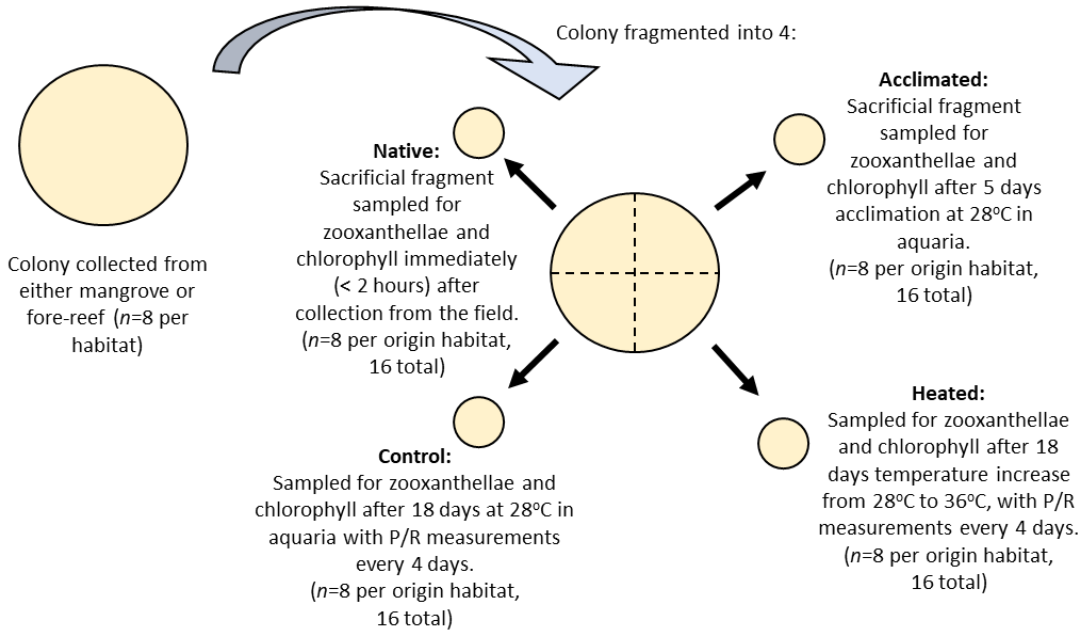

Figure 2.3. Schematic of experimental design depicting collection of colonies (*n* = 8 per habitat), fragmentation (*n* = 4 per colony), assignment to treatment (*n* = 8 per habitat, per treatment) and sacrificial sampling of native, acclimated, control and heat stressed corals.

2.2.7. Productivity vs respiration

Net primary productivity and respiration were measured at each temperature (28, 30, 32, 34 and 36˚C) by change in dissolved oxygen (DO) concentration following incubation of coral fragments in light and dark conditions, respectively. Coral fragments were transferred to 500 ml sealed transparent histology bottles, each containing a magnetic stir bar to ensure homogeneity of DO, and within a water bath set to their respective treatment temperature during all dissolved oxygen measurements (Fig. 2.4). Corals were left to acclimatise in the bottles for 10 minutes before the first reading was taken. Bottles were left for an incubation period of 30 minutes in light (300 µmol photons m^{-2} s^{-1}) followed by 30 minutes in darkness. Measurements of DO were taken at 10 second intervals throughout each incubation period using a Vernier Optical DO Probe connected to a LabQuest Mini (Vernier) and recorded using the LoggerLite software (Vernier). Net primary productivity (NPP) and respiration (R) of each coral was calculated by plotting linear regressions and taking the slope of each line as

the rate per minute. Gross primary productivity (GPP) was calculated by adding R (oxygen consumption) to NPP (net oxygen evolution). Gross primary productivity: respiration ratios (P/R) were calculated by dividing GPP by R.

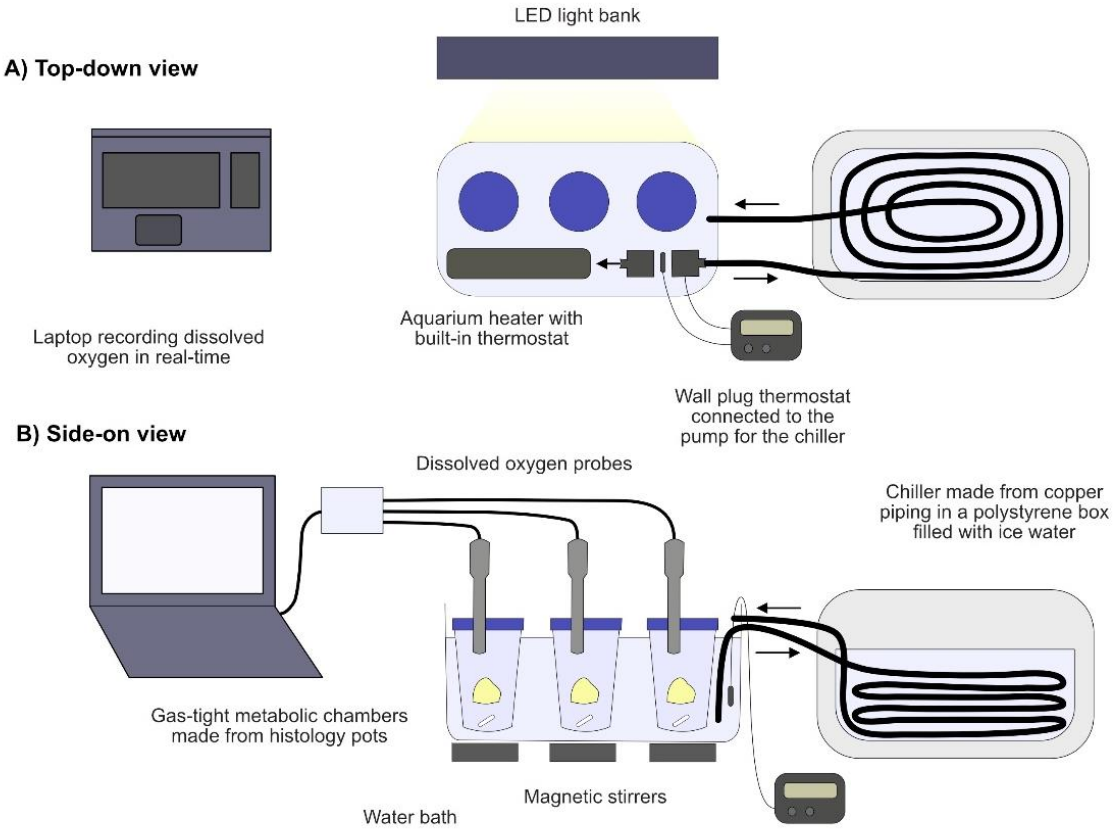

Figure 2.4. Diagram of the field-friendly, cost-effective metabolic chamber set-up. **A)** Top-down view **B)** Side-on view. Coral fragments were enclosed within 500 ml transparent, gas-tight metabolic chambers, each containing a magnetic stir bar to ensure homogeneity of dissolved oxygen. The temperature of the water bath was controlled using an aquarium heater with a built-in thermostat, and a custom-made chiller. Water from the water bath was recirculated through an ice box, regulated by a wall-plug thermostat. Light and dark conditions for measuring net primary productivity and respiration respectively, were achieved using an LED light bank. Dissolved oxygen was measured in real-time using a series of three probes connected to a laptop.

To elucidate whether any decrease in P/R ratio was due to a decrease in productivity or an increase in respiration, NPP and R were corrected for surface area of the coral fragments so that final values were expressed as [DO]mg ml^{-1} cm^{-2} h^{-1}. Surface area was measured using a non-destructive foil coverage method (Marsh, 1970) whereby live coral fragments were totally covered in aluminium foil ensuring no overlap, and this foil was weighed. The surface

area was then calculated based on the mass of a known area of foil. Foil-wrapping is a relatively accurate method for measuring surface area of massive-morphology *Porites* spp. (Veal *et al.*, 2010).

2.2.8. Algal symbiont density

Coral tissue was removed from each fragment using a Waterpik (Waterpik Inc, England) in approximately 10 ml of filtered seawater (FSW); the exact volume of FSW was noted and area of tissue removed was calculated using ImageJ. The resulting tissue slurry was homogenised using a Pasteur pipette and a 2 ml aliquot taken for cell quantification via microscopy using a Neubauer haemocytometer (Berkelmans & van Oppen, 2006).

2.2.9. Chlorophyll concentration

Pigments were extracted from coral tissue in 1 ml 100% methanol at 4°C for 24 h (Jeffrey & Haxo, 1968). Methanol was chosen for its efficient extraction of pigments from recalcitrant samples, which permitted extraction from whole coral tissue (Porra, 1989). The coral tissue was scraped from the skeleton using sterile forceps (to minimise endolithic algae contribution to chlorophyll concentration), and the surface area of tissue removed was calculated using ImageJ. Extracts were stored in sealed cryovials in the dark at -20°C before chlorophyll *a* quantification by spectrophotometer at 665 nm using Ritchie's (2008) coefficients. Corrections were made for turbidity, measured at 750 nm, and degradation of samples was accounted for by acidifying samples to a final concentration of 0.003M HCl to break down chlorophyll to phaeopigments, again measured at 665 nm (Holm-Hansen & Riemann, 1978).

2.2.10. Statistical analyses

All analyses were carried out using R 3.4.1 (R Core Team 2017). Differences in coral metabolism (P/R, GPP, and R) were tested separately with respect to habitat and thermal treatment using linear mixed-effects models (LMM) with the `lme4` R package (Bates *et al.*, 2015). Habitat, heating regime, and time were modelled as fixed factors, with levels reef vs. mangrove and control vs. heated, respectively, in addition to their interactions. Random factors were modelled to account for the experimental design; coral colony identity, implicitly nested within habitat, was modelled with random intercepts. Repeated measures of each coral fragment were initially modelled by specifying fragment identity as a random factor across time. However, there was no random effect of fragment identity, so this term was dropped from the final model. Likelihood ratio tests and Akaike's information criterion (AIC) were used to compare models with random slopes and intercepts to random intercepts-only.

Differences in algal symbiont density and chlorophyll *a* concentration, measured at the end of the experiment, between control and heated corals were tested using generalised linear models (GLMs). There were two fixed factors: habitat and thermal treatment, each with two levels: reef vs. mangrove and control vs. heated, respectively. Although each coral colony was split between treatments (paired design), there were not enough observations to support a mixed-effects model with colony identity as a random factor. Models were specified with the best fitting link function to account for distribution and dispersion of the data. Assumptions of normality, and heteroscedasticity (equal variances), were assessed by graphical inspection of each model's residuals. Post-hoc pairwise comparisons were made using Tukey's Honest Significant Differences test.

2.3. Results

2.3.1. Productivity vs respiration

After the five-day acclimation period, corals exhibited P/R ratios above 1.5, signifying oxygen evolution (primary productivity) being greater than oxygen consumption (respiration) (Fig. 2.5 A). This ratio increased across all treatments over the next 4 days by at least 12%. The P/R ratio of *P. lutea* subjected to increasing temperature declined by over 70% from day 8 (30 °C) until the end of the experiment on day 20 (36 °C), regardless of whether the coral originated from fore-reef or mangrove habitat. Thus, there was a highly significant negative effect of heating over time ($\beta_{heating:time}$ = -0.095, SE = 0.018, t(158) = -5.186, $P < 0.001$), but no significant effect of habitat ($\beta_{habitat}$ = 0.207, SE = 0.278, t(158) = 0.746, $P > 0.05$), and thus no interaction effect ($\beta_{heating:habitat:time}$ = 0.003, SE = 0.026, t(158) = 0.098, P > 0.05). Corals kept under ambient temperature (28 °C) for the duration of the experiment showed no significant change in P/R over time (β_{time} = 0.014, SE = 0.013, t(158) = 1.085, $P > 0.05$; Table 2.1; Fig. 2.5 A).

At the start of the experiment, mangrove corals exhibited higher GPP than reef corals ($\beta_{habitat}$ = 0.032, SE = 0.013, t(158) = 2.388, $P < 0.05$), which remained consistent for corals kept in the control treatment throughout the experiment (β_{time} = -0.001, SE = 0.001, t(158) = -1.275, p > 0.05). However, the GPP of all corals subjected to increasing heat stress decreased by at least 82% over the course of 16 days ($\beta_{heating:time}$ = -0.003, SE = 0.001, t(158) = -4.058, $P < 0.001$). The GPP of mangrove-origin corals decreased rapidly by 59% between days 12 to 16 (Fig. 2.5 B), corresponding to a rise in temperature of the heated aquaria from 32°C to 34°C (Fig. 2.2). From the sea surface temperature time series (Fig. 2.1 inset), it is apparent that mangrove corals regularly experience temperatures of 34°C between the months of November and February, and even survive peak temperatures in nature nearing 38°C.

However, during the cooler months, when the heat-ramping experiment was conducted, Langira mangrove rarely reaches 31°C.

Respiration rates were not affected by heating over the course of the experiment ($\beta_{heating:time}$ = -0.001, SE = < 0.001, t(158) = -1.458, $P > 0.05$). Though there was a weak laboratory acclimation effect (β_{time} = -0.001, SE = < 0.001, t(158) = -2.527, $P < 0.05$; Fig. 2.5 C).

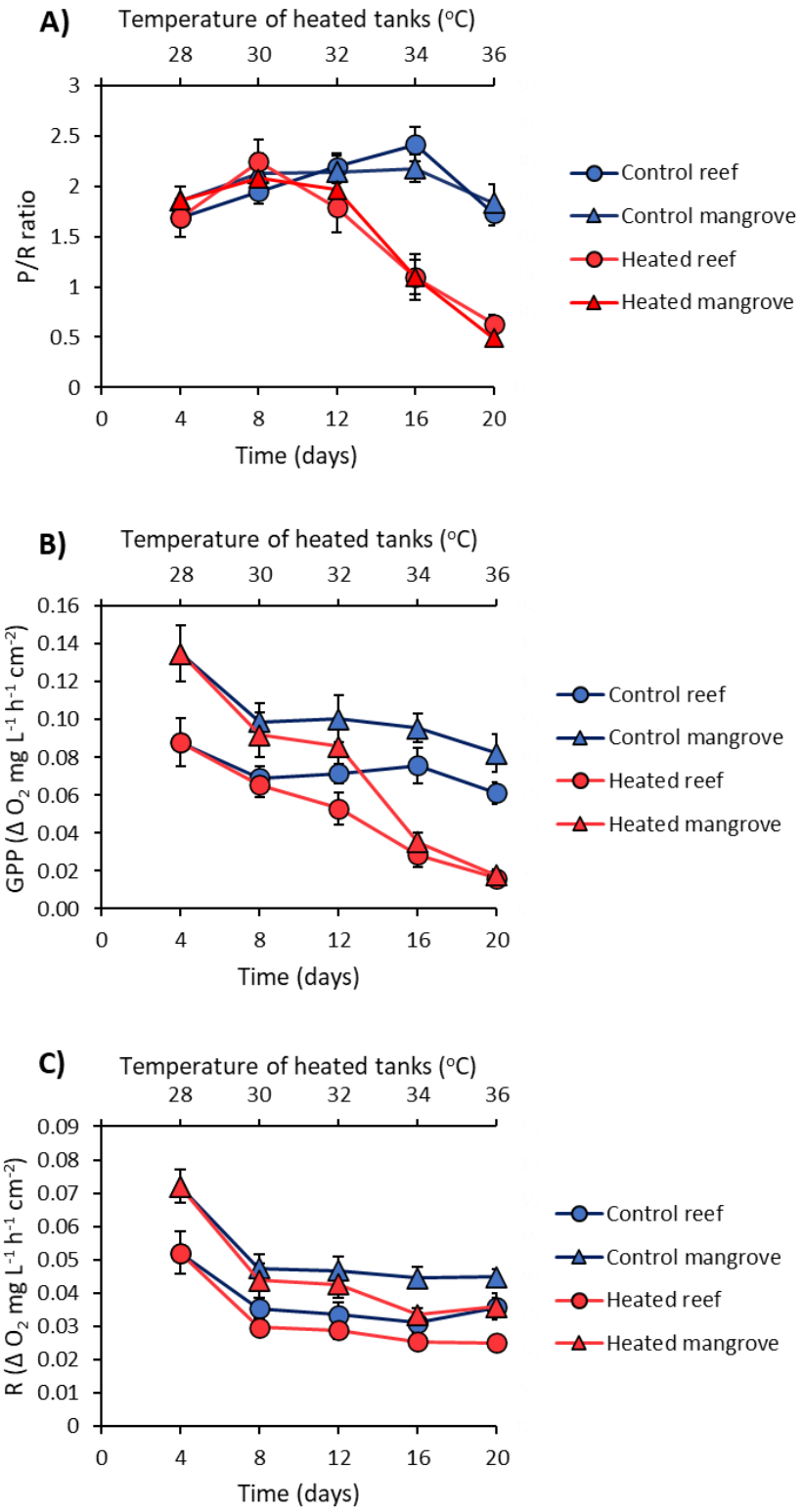

Figure 2.5. A) Productivity vs respiration. Values are mean P/R ratios ± SE reflecting GPP divided by R (n = 8). Values of P/R ratio > 1 represent productivity being greater than respiration, whereas < 1 reflect coral holobionts respiring more than photosynthesising. **B)** Gross primary productivity. **C)** Respiration. Values are mean change in dissolved oxygen concentration per hour per cm^2 coral tissue ± SE (n = 8). Control aquaria were maintained at 28°C throughout the experiment. Heated aquaria were ramped from 28°C to 36°C over 15 days, starting at day 5 (see Fig. 2.2 for heating regime).

Table 2.1. Results of linear mixed-effects models (LMMs) † and generalised linear models (GLMs) †† for each response parameter. Algal symbiont density (count data) based on poisson distribution and *ln* link function. All other models based on gaussian (normal) distribution.

Response parameter	Factor	Fixed effects estimate (β)	Test statistic	Df	*n*	p-value
P/R ratio †	~ Treatment × Habitat × Time + (1 | Colony)					
	heating regime	**0.639**	**2.621**			**< 0.01**
	habitat	0.207	0.746			0.465
	time	0.014	1.085			0.278
	heating × habitat	-0.056	-0.161			0.872
	heating × time	**-0.095**	**-5.186**			**< 0.001**
	habitat × time	-0.015	-0.778			0.437
	heating × habitat × time	0.003	0.098		158	0.922
Gross Primary Productivity (GPP) †	~ Treatment × Habitat × Time + (1 | Colony)					
	heating regime	0.018	1.599			0.110
	habitat	**0.032**	**2.388**			**< 0.05**
	time	-0.001	-1.275			0.202
	heating × habitat	0.009	0.555			0.579
	heating × time	**-0.003**	**-4.058**			**< 0.001**
	habitat × time	-0.001	-0.649			0.516
	heating × habitat × time	-0.001	-1.064		158	0.287
Respiration (R) †	~ Treatment × Habitat × Time + (1 | Colony)					
	heating regime	1.223	0.242			0.808
	habitat	**1.105**	**1.933**			**0.053**
	time	**-6.797**	**-2.527**			**0.011**
	heating × habitat	8.994	0.125			0.900
	heating × time	-5.544	-1.458			0.144
	habitat × time	3.248	0.084			0.932
	heating × habitat × time	-9.250	-0.170		158	0.865
Algal symbiont density ††	~ Thermal × Habitat (family = quasipoisson)					
	heating regime	**-0.761**	**-3.539**	**1**		**0.001**
	habitat	0.628	4.176	1		**<0.001**
	heating × habitat	0.373	1.454	1	32	0.16
Chlorophyll *a* per cm^2 ††	~ Thermal × Habitat (family = gaussian)					
	heating regime	**-2.247**	**-2.805**	1		**< 0.01**
	habitat	**3.773**	**4.709**	1		**< 0.001**
	heating × habitat	**1.700**	**2.121**	1	32	**< 0.05**
Chlorophyll *a* per symbiont cell ††	~ Thermal × Habitat (family = gaussian)					
	heating regime	-0.884	-1.867	1		0.072
	habitat	0.394	0.833	1		0.412
	heating × habitat	0.844	1.260	1	32	0.218

†LMMs for repeated measures with multiple time points (P/R ratio, GPP, R)

††GLMs for end point comparisons (symbiont density, and chlorophyll *a* concentration)

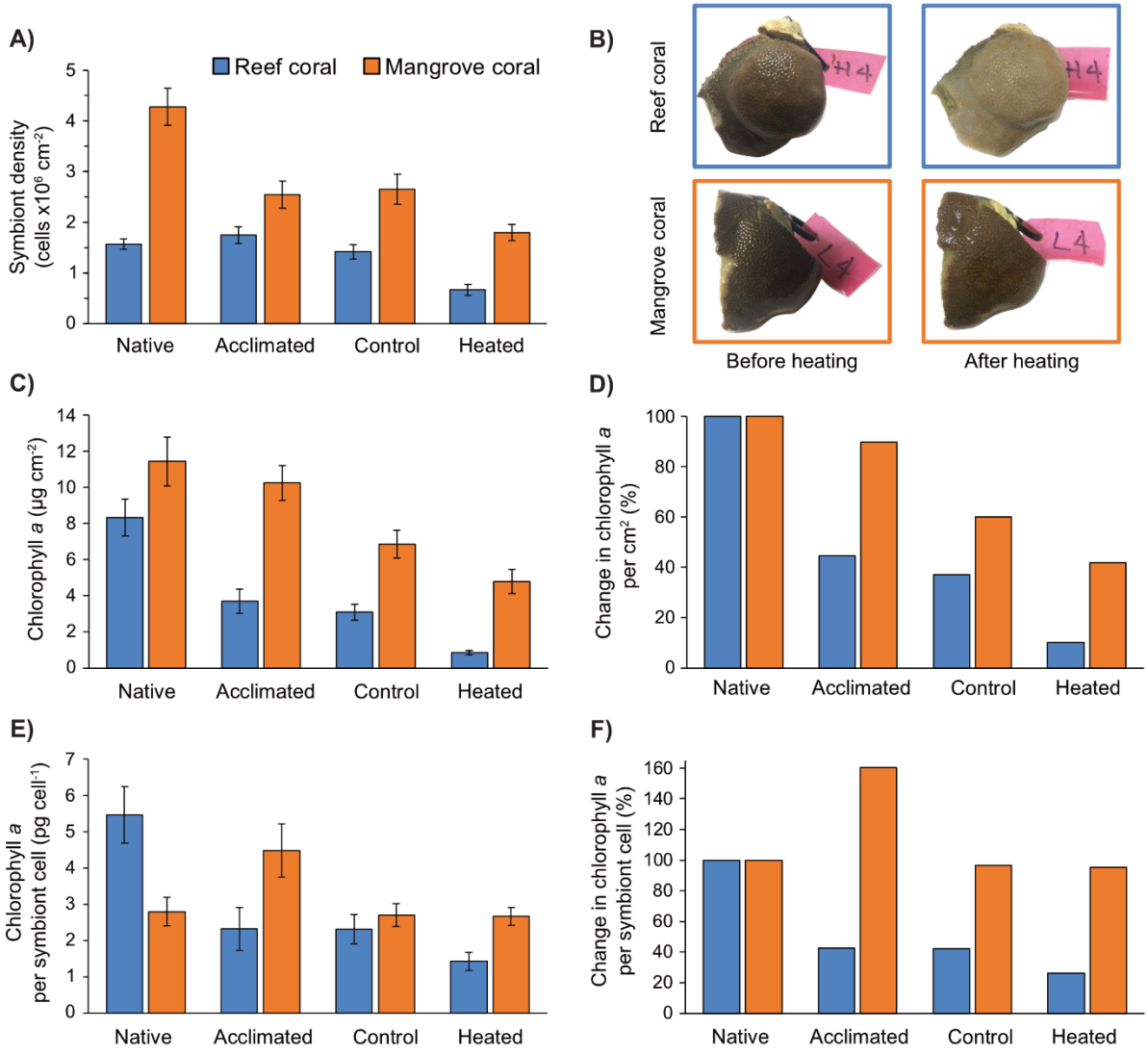

Figure 2.6. A) Symbiont density per cm^2 of coral tissue. **B)** Photographs of the same fragment taken before and after heat treatment. **C)** Chlorophyll *a* content per cm^2 of coral tissue. **D)** Percentage change in chlorophyll *a* per cm^2. **E)** Chlorophyll *a* content per symbiont cell. **F)** Percentage change in chlorophyll *a* per symbiont cell. Values are mean ± SE (*n* = 8). Reef corals shown in blue; mangrove corals in orange. Native samples were taken directly after collection from the field, acclimated samples were taken after 5 days of acclimation under laboratory conditions, control and heated samples were taken at the end of experiment on day 18.

2.3.2. Algal symbiont density

Mangrove corals hosted higher Symbiodiniaceae densities than reef corals both *in situ* (by 173%) and throughout the experiment (by at least 46%). There was no difference in symbiont densities between native, acclimated, and control reef corals, while heat-stressed

reef corals exhibited reduced symbiont densities. Symbiodiniaceae densities of mangrove corals decreased by 41% when acclimated to outdoor laboratory conditions, but remained constant under control treatment. At the end of the experiment, the symbiont densities of reef corals subjected to heat stress averaged 53% lower than controls kept at 28°C, while heat-stressed mangrove corals hosted only 32% lower symbiont densities than controls (Fig. 2.6 A). There was a clear difference in symbiont density dependent on habitat ($\beta_{habitat}$ = 0.63, SE = 0.15, t(32) = 4.18, $P < 0.001$), as well as heating ($\beta_{heating}$ = -0.76, SE = 0.22, t(32) = -3.54, $P < 0.01$). However, there was no interaction between habitat and heat stress ($\beta_{heating:habitat}$ = 0.37, SE = 0.26, t(32) = 1.45, $P > 0.05$) as both mangrove and reef corals experienced declines in symbiont density with heat stress.

2.3.3. Chlorophyll concentration

The concentration of chlorophyll *a* per area of coral tissue generally followed the same pattern as symbiont density, with corals from the mangroves containing at least 37% more chlorophyll *a* than reef corals (Fig. 2.6 A, C & D; $\beta_{habitat}$ = 3.77, SE = 0.80, t(32) = 4.71, $P < 0.001$). Chlorophyll *a* content of both mangrove and reef-origin corals decreased between native (samples taken immediately after collection), acclimated (samples taken after 5 days in aquaria), and control samples (taken at the end of experiment on day 18), with heating exacerbating this trend. While there was a significant effect of thermal stress on chlorophyll *a* concentration ($\beta_{heating}$ = -2.25, SE = 0.80, t(32) = -2.81, $P < 0.01$), post-hoc testing showed there was no significant difference in mean chlorophyll *a* concentration between control mangrove corals (6.86 ± 0.77 µg chlorophyll *a* cm^{-2} coral tissue) and heated mangrove corals (4.79 ± 0.68 µg cm^{-2}; $P > 0.05$). By day 18, the chlorophyll *a* content (standardised to area) of reef corals subjected to heat stress (0.84 ± 0.13 µg cm^{-2}) was, on average, 73% lower than controls kept at 28°C (3.09 ± 0.43 µg cm^{-2}; $P < 0.05$), while heat-stressed mangrove corals exhibited only 30% lower chlorophyll concentration than controls,

concordant with algal symbiont losses (Fig. 2.6 A, C & D). More severe bleaching was measured in reef corals, relative to mangrove corals (Fig. 2.6 B), as a result of a 38% difference in chlorophyll *a* concentration *per symbiont cell* between heated and control treatments of reef corals (Fig. 2.6 E & F; Table 2.1; $\beta_{heating}$ = -0.88, SE = 0.47, t(32) = -1.87, *P* = 0.07).

2.4. Discussion

This study was the first to test the thermal tolerance of corals living in a highly thermally variable mangrove habitat. Here, the experimental results show that *P. lutea* naturally occurring in a thermally variable mangrove habitat were more resistant to bleaching than conspecifics from a fore-reef environment (Fig. 2.6). However, superior thermal tolerance was not reflected in terms of coral holobiont metabolism since corals exhibited similar heat-induced declines in productivity regardless of habitat (Fig. 2.5). While corals from the mangrove can survive thermal regimes which would otherwise bleach corals from typical reef habitats, all corals in this study were susceptible, in terms of productivity, to heating exceeding their regional bleaching threshold of 32°C for 8 days (Fig. 2.1 inset, Fig. 2.2 & 2.5 B), similar to findings from the thermally extreme Kimberley region of northwest Australia (Schoepf *et al.*, 2015).

Despite the large differences in the range of temperatures naturally experienced by *P. lutea* originating from mangrove versus fore-reef habitat, mangrove-origin corals showed no difference in P/R ratio compared with reef-origin corals when subjected to increasing temperature (Fig. 2.5 A). This contrasts to previous findings whereby corals (*Montastraea annularis)* originating from inner lagoon sites characterised by high daily thermal maxima, exhibited higher P/R ratios than conspecifics from outer barrier reef sites with lower maximum temperatures, when exposed to elevated temperature treatments between 29 and 35°C (Castillo & Helmuth, 2005). In the current study, *P. lutea* from both habitats showed a

decreased ratio of P/R when subjected to temperature increases, due to decreased productivity (Fig. 2.5). It is well documented that heat stress often results in reduced coral holobiont productivity due to accumulation of free radicals damaging the algal symbionts' photosystems (Weis, 2008). Taken in isolation, these results suggest that there is no real advantage gained by living in the extreme conditions of mangrove habitats, or at least that any thermal resistance gained is not readily transferable to other settings. This raises pertinent questions about whether these corals are locally adapted to stressful conditions, or whether environmental conditions in the mangroves permit survival in spite of other stressors. Corals found living in the mangroves of Bouraké, New Caledonia, were found to naturally exhibit P/R ratios less than 1 *in situ*, so would have effectively been existing in deficit were they not making up for their energy requirements through heterotrophy (Camp *et al.*, 2017)**.**

Whilst the metabolic activity of the coral holobiont might suggest there is no difference in thermal tolerance limits between mangrove and fore-reef corals, symbiont physiology provides an alternate conclusion. *Porites lutea* originating from the mangrove habitat consistently hosted higher symbiont densities and chlorophyll concentrations than corals originating from the fore-reef habitat, regardless of temperature treatment, indicating differences in physiological strategy. This was an unexpected result for corals from a thermally fluctuating environment with high extreme temperatures, since it is widely regarded that hosting excess algal symbionts increases the risk of bleaching (Nesa & Hidaka, 2009; Cunning & Baker, 2013). The difference in symbiont densities and chlorophyll concentrations of native coral samples could be explained by greater nutrient loading in the mangroves, as nitrogen is known to drive increased Symbiodiniaceae densities (Falkowski *et al.*, 1993; Fabricius, 2005). This is supported by the subsequent decrease in mangrove-origin symbiont densities following acclimation to aquaria (Fig. 2.6 A). However, symbiont density and chlorophyll content of mangrove-origin corals does not decrease to the same levels as those

of their reef-origin counterparts, neither following the 5-day acclimation period, nor in controls on day 18, as would be expected during photo-acclimation to the same light environment (Falkowski & Dubinsky, 1981; Roth *et al.*, 2010). This suggests that the mangrove-origin corals are generally more resilient to bleaching, be that due to thermal stress, or just being housed in aquaria, when compared with their reef-origin counterparts.

As well as a clear difference in heat-induced bleaching susceptibility between corals from mangrove versus fore-reef habitat (Fig. 2.6), the mechanism of bleaching also differed dependent on habitat. Heat-stressed reef corals bleached more severely through loss of symbiont cells as well as reduction in chlorophyll *a* per cell, whereas mangrove corals bleached to a lesser degree through only symbiont loss. Such habitat-dependent differences in bleaching mechanism are corroborated by previous findings (Hoegh-Guldberg & Smith, 1989; Warner *et al.*, 1996; Schoepf *et al.*, 2015). This includes findings from Ofu Island, American Samoa, where corals native to a high temperature variation back-reef pool retained more chlorophyll following experimental heat stress than corals transplanted into the same pool, which retained more chlorophyll than corals transplanted into a moderate temperature variation pool (Palumbi *et al*., 2014).

An advantage of conducting common-garden experiments is that confounding environmental factors can be disentangled. In this study, in the absence of environmental conditions present in the mangrove, the mangrove-dwelling corals did not fare much better (at least metabolically) under heat stress, than their reef-dwelling counterparts. Ergo, there must be something in the mangrove which allows their metabolic strategy to succeed. The mangrove corals regularly experience temperatures of the magnitudes tested here (Fig. 2.1), yet P/R ratio dropped below 1 following heat stress (representing a shift away from net productivity and thus the cost of respiration no longer being covered by productivity; Fig. 2.5). Therefore, this physiological strategy might *only* succeed *in* the mangroves, where a switch to heterotrophy can be made. This theory would, however, rely on the provision of enough

suspended particulates for the coral host to consume, and avoid symbionts becoming parasitic, as has been documented in abundance of nutrients (Baker *et al.*, 2018). These are, of course, just ideas which warrant testing. Nevertheless, there is precedent, since *P. lutea* has previously been shown to acclimate to extreme changes in temperature and pH through changing its polyp expansion behaviour and photosynthetic efficiency, thereby modulating heterotrophy and autotrophy (Pacherres *et al.*, 2013). A congener, *P. lobata*, has also been shown to exhibit phenotypic plasticity (specifically in skeletal growth, density, and calcification) in response to habitat type (Smith *et al.*, 2007). It should also be noted that *P. lutea* is well known to be a stress tolerant species with previously reported survival at temperatures as low as 13°C (Chen *et al.*, 2016), and as high as 36°C (Sheppard *et al*., 1992). A key limitation of this study, and many other heat ramping experiments, is whether heat ramping can provide a true estimation of thermal tolerance, since corals will inevitably carry over a 'hangover' from the previously accumulated heat stress. Another consideration is whether laboratory and aquaria studies can ever be considered representative of natural warming events.

Together these results suggest that living in a mangrove may offer only modest pre-conditioning to corals under warming scenarios in other settings. Similar conclusions were drawn from a study on *Porites astreoides* in marginal seagrass habitat in the Caribbean (Camp *et al.*, 2016a). Corals from high variability seagrass habitat showed no enhanced tolerance compared with corals from low variability reef habitat when exposed to superimposed predicted future climate conditions – the impact of elevated temperature and/or pH on calcification and metabolic rates was the same regardless of habitat (Camp *et al.*, 2016a). While exposure to highly variable temperatures can enhance coral resistance to thermal bleaching (Middlebrook *et al.*, 2008; Oliver & Palumbi, 2011a), it does not render coral invincible to extreme marine heatwaves, that are expected to become more frequent in coming years. Several studies have concluded that even naturally heat-resistant coral

populations have rigid thermal limits between 1-3°C above their regional summer maximum, leaving them vulnerable to ocean warming (Middlebrook *et al.*, 2008; Coles & Riegl, 2013; Schoepf *et al.*, 2015, 2019).

While not all marginal habitats may prepare corals for future climate scenarios, their extreme conditions do exhibit potential for coral acclimatisation and/or adaptation. If corals have become locally adapted to these extreme environments through natural selection, they could represent reservoirs of stress-resistant genetic diversity. Marginal habitats have also been studied for their potential as climate refugia, with buffers against unfavourable future conditions (Camp *et al.*, 2018). Corals from these habitats may end up being survivor stocks, and could be important for re-seeding degraded reefs. Also, corals from naturally thermally 'extreme' or highly variable habitats could be used for active coral restoration with the aim of farming corals for climate resilience (Morikawa & Palumbi, 2019). However, it would be prudent to test the thermal limits of corals from extreme environments under a range of thermal regimes, and in a variety of controlled aquaria and field settings, before using them as a stock for active restoration.

No marginal habitat can provide a perfect analogue to future reefs, though they do represent a useful tool for understanding the physiological limits of corals in a natural setting (Camp *et al.*, 2018). Marginal habitats alone are not the solution to the destruction of coral reefs by anthropogenic climate change; without question our priority to save coral reefs must be on cutting emissions of greenhouse gases (Van Hooidonk *et al.*, 2013, 2016). So, it is important that decision makers not view these glimmers of hope for corals as catch-all solutions, thereby providing excuses not to drastically curb emissions. Results presented here contribute the first piece of evidence toward understanding marginal mangrove coral thermotolerance, which, in turn could inform management/mitigation options to the impacts of marine heatwaves on coral reefs.

2.5. Acknowledgements

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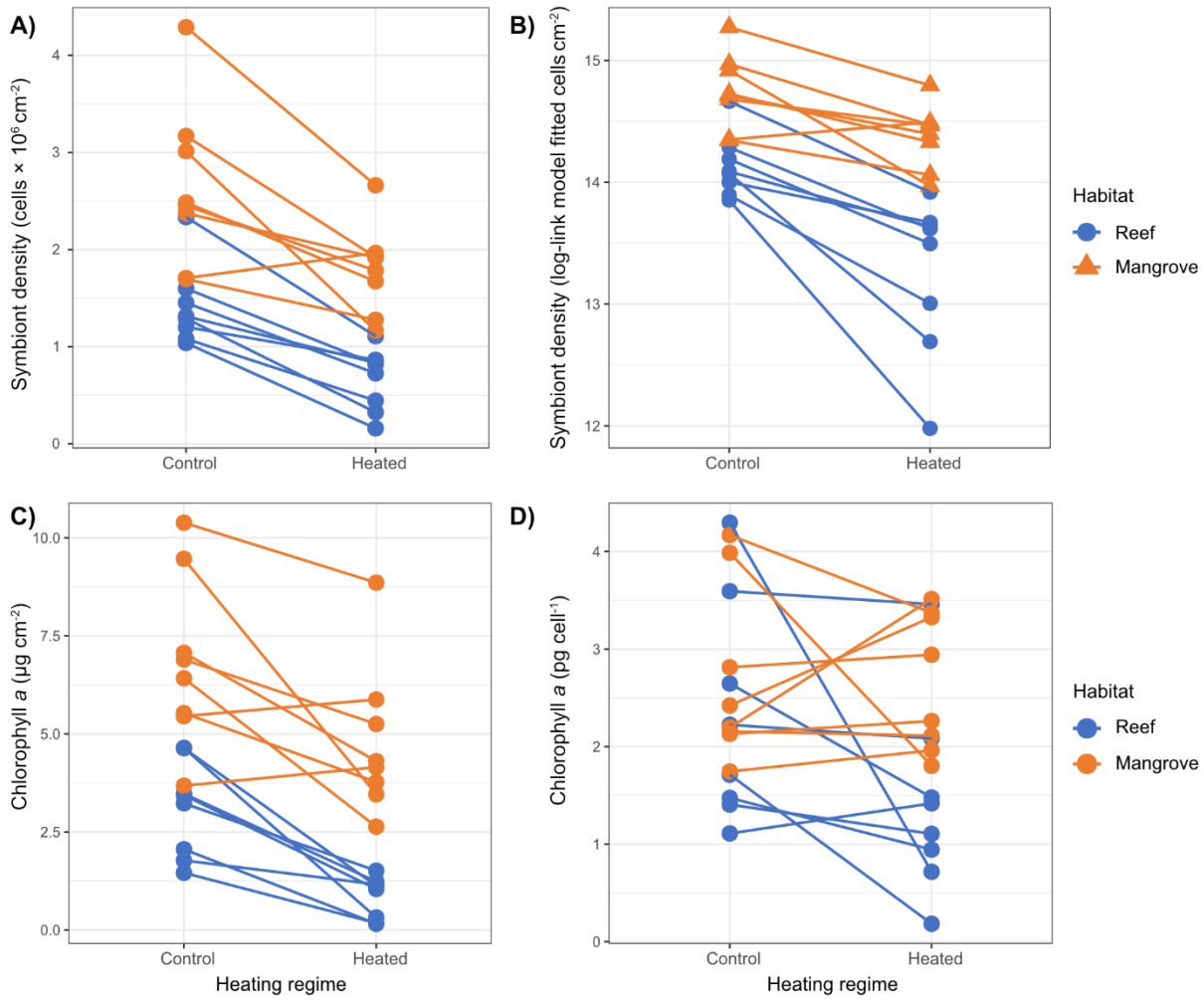
2.7. Supplementary material



Supplementary figure 2.1 A) Symbiont density per cm^2 of coral tissue. **B)** Model fitted values for symbiont density. **C)** Chlorophyll *a* content per cm^2 of coral tissue. **D)** Chlorophyll *a* content per symbiont cell. Points denote individual data points, joined by a line for colony identity because colonies were split between treatments (paired design). Reef corals shown in blue; mangrove corals in orange.

Chapter 3: The response of coral holobionts to reef - mangrove reciprocal translocations

Abstract

Scleractinian corals associate with a broad array of microorganisms, forming a meta-organism termed the coral holobiont. While coral holobionts are known to change in response to environmental conditions, little is known about the holobionts of mangrove- versus reef-dwelling corals. Therefore, reciprocal translocations of the reef-building coral, *Porites lutea*, within Curieuse Marine National Park, Seychelles, sought to address whether the abundance, diversity, and composition of the coral microbiomes differed between mangrove and reef habitats, and whether they could flexibly reorganise based on the prevailing habitat. Amplicon sequencing of coral-associated bacteria and Symbiodiniaceae revealed that the bacterial community composition of *Porites lutea* was habitat-driven and highly flexible, while the algal symbionts were habitat-influenced but showed greater host-fidelity, remaining more stable over time. Hahellaceae which contains the known bacterial endosymbiont, *Endozoicomonas*, dominated the bacterial assemblage of *Porites lutea* from both habitats. However, corals from the mangrove also featured high relative abundances of Rhodobacteraceae (14%), Flavobacteriaceae (10%), Alteromonadaceae (6%), and Vibrionaceae (6%) – taxa sometimes linked to diseased coral. Within 20 hours of translocation to a new habitat, the once distinct coral-associated bacterial communities had become highly similar. It is not known whether the habitat-distinctive microbial communities hosted by *Porites lutea* aid coral survival and promote local adaptation to specific habitats or whether the assemblages are opportunistic. There was little evidence of local adaptation as all corals survived translocations of one year, though other trade-offs should be studied.

Such rapid reorganisation of coral-associated bacterial communities continues to provide hope as an adaptive strategy to survive fast-changing environmental conditions.

3.1. Introduction

Scleractinian corals associate with a wide array of microorganisms, including endosymbiotic algae (Symbiodiniaceae), fungi, protists, bacteria, archaea, and viruses, which together form the meta-organism termed the 'coral holobiont' (Rohwer *et al.*, 2002; Zilber-Rosenberg & Rosenberg, 2008). The dependence of reef-building corals upon the energy derived from their photosynthetic algal symbionts is well-documented (Yellowlees *et al.*, 2008), while the functions performed by other coral-associated microorganisms represent a rapidly advancing field of study (Bourne *et al.*, 2016). Recently attributed microbial roles include provision of otherwise unavailable nutrients and vitamins to the coral host through microbial carbon pathways (Kimes *et al.*, 2010), nitrogen fixation (Lema *et al.*, 2012; Bourne *et al.*, 2016), and dimethyl-sulfoniopropionate (DMSP) metabolism (Raina *et al.*, 2009, 2010), which can be extremely important in oligotrophic environments such as reefs. Having originally been implicated with causing disease (Kushmaro *et al.*, 1996), coral-associated bacteria are now also known to provide a first line of defence to the coral through both the active production of antimicrobials (Raina *et al.*, 2016), and indirect prevention of colonisation by opportunistic pathogens (Ritchie, 2006; Shnit-Orland & Kushmaro, 2009; Krediet *et al.*, 2013). There is also evidence of a 'core microbiome' which is associated with almost all corals, and likely provides many essential, but as yet unknown, functions (Ainsworth *et al.*, 2015; Hernandez-Agreda *et al.*, 2016b).

Coral-associated microbial communities are dynamic and known to differ with biogeography (McKew *et al.*, 2012), habitat type (Pantos *et al.*, 2015), and coral host species (Kvennefors *et al.*, 2010), as well as spatially within corals (between the mucus, tissue and skeleton; Sweet *et al.*, 2011b), and temporally with season (Koren & Rosenberg, 2006), tidal flux

(Sweet *et al.*, 2017b), and coral colony age (Williams *et al.*, 2015). Several sequencing studies have shown the coral microbiome to be regulated, and potentially selected for, by environmental conditions (Ziegler *et al.*, 2016, 2019; Camp *et al.*, 2020). For example, in the Red Sea, a higher abundance of opportunistic bacterial families, such as Vibrionaceae and Rhodobacteraceae, typified corals from sites more impacted by anthropogenic input, despite the corals appearing healthy (Ziegler *et al.*, 2016). Recent evidence is mounting which suggests that the bacterial portion of the coral microbiome is more environmentally influenced than host-regulated (Osman *et al.*, 2020).

As environmental conditions continue to shift with the increasing pace of global climate change (Veron *et al.*, 2009; Heron *et al.*, 2016) and growing human demands on coastal habitats (Jackson *et al.*, 2001; Hughes *et al.*, 2003), long-lived, sessile corals are becoming ever more threatened. The visible effects of anomalously high sea surface temperatures (SSTs) on corals are well known; one study found that 75% of globally distributed reef sites surveyed had bleached during the most recent record-breaking global marine heatwave of 2016 (Hughes *et al.*, 2018a). While the dysbiosis of coral host and algal symbionts due to adverse environmental conditions is clear to see, the environmental impacts on the rest of the coral microbiome can go unnoticed. Nevertheless, the changes to the coral-associated bacterial community brought about by environmental change can be profound (Bruno *et al.*, 2007; reviewed in Fry *et al.*, 2020). Elevated temperatures can initiate pathogenesis in coral microbiomes (Rosenberg & Ben-Haim, 2002; Vega Thurber *et al.*, 2009), leading to impaired microbial functions, or a disease-associated state recently referred to as the ‘pathobiome’ (Sweet & Bulling, 2017).

Despite the decline of coral reefs worldwide (Gardner *et al.*, 2003; Bruno & Selig, 2007), there are pockets of seemingly super-tolerant corals living under extreme temperature, pH, and dissolved oxygen conditions in so-called marginal habitats (Camp *et al.*, 2018; and detailed in Chapter 2). Furthermore, the bacterial and Symbiodiniaceae communities hosted

by the corals living under extreme conditions are different to those of conspecific corals residing on neighbouring reefs (Camp *et al.*, 2020). Different Symbiodiniaceae have different environmental niches and tolerances (Sampayo *et al.*, 2007; Grégoire *et al.*, 2017), which have a bearing on the coral holobiont's ability to withstand environmental extremes (Baker *et al.*, 2004; Iglesias-Prieto *et al.*, 2004; Hoadley *et al.*, 2019). Habitat-dependent differences in coral-associated bacterial assemblages have also been linked to the thermal tolerance of the coral host (Ziegler *et al.*, 2017). However, there is a debate over whether the bacterial community conferred fitness to the coral holobiont, or whether the same high-temperature selection pressure acted simultaneously on both coral and bacterial community. The 'coral probiotic hypothesis' – the notion that the microbiome could aid coral in adapting to new environmental conditions – was first coined over a decade ago (Reshef *et al.*, 2006), and has since been a popular but enigmatic research topic. Theoretical support and potential mechanisms for how the microbiome could provide a rapid means of coral holobiont adaptation have been reviewed on numerous occasions (Torda *et al.*, 2017; Fry *et al.*, 2020; Voolstra & Ziegler, 2020), but there is not yet any unequivocal empirical evidence.

A study of the depth-generalist coral *Pachyseris speciosa* showed there to be a number of bacteria consistently associated with corals from mesophotic reefs, as well as a core microbiome present across all depths, and a portion of the bacterial community which was highly variable (Hernandez-Agreda *et al.*, 2016a). There is a paucity of information regarding the diversity and composition of microorganisms associated with corals from mangrove environments. One recent study has hypothesised that differences in the microbiomes of corals from reef and mangrove habitats may support coral holobiont productivity, and therefore the ability to survive under the extreme temperature, pH, and dissolved oxygen fluctuations of mangroves (Camp *et al.*, 2020). However, it remains to be seen whether there is a specific mangrove or marginal habitat-associated coral microbiome. And, despite a wealth of sequencing studies demonstrating coral microbiome flexibility (Ziegler *et al.*, 2019;

Osman *et al.*, 2020; Röthig *et al.*, 2020), the time scales of microbial turnover are not certain, and are not often the focus of such studies.

To assess whether the abundance, diversity, and composition of the coral microbiome differs between marginal and reef habitats, a reciprocal translocation experiment of *Porites lutea* was implemented in Curieuse Marine National Park, Seychelles. This study was conducted one year following the catastrophic mass-bleaching event of 2016, which saw greater proportional bleaching and mortality of corals in some habitats over others (Gardner *et al.*, 2019). The experimental design allowed the assessment of whether, and over what time scale, the microbial community would be able to reorganise in response to a new habitat with new environmental conditions. Results of similar translocation experiments have been based on sampling at least a year after transplantation (17 months: Ziegler *et al.*, 2017; 21 months: Ziegler *et al.*, 2019), missing the key early colonisation and successional stages. Therefore, microbial communities of *Porites lutea* cross- and back-transplanted into both reef and mangrove habitats were characterised using amplicon sequencing to quantify early changes in microbial composition, as well as the longer-term microbial community changes after one year in a new environment.

3.2. Methods

3.2.1. Habitat characterisation

To characterise the fore-reef habitat known as Home Reef (Anse Papaie; 4° 17' 05.1" S, 55° 44' 07.6" E), and mangrove-influenced habitat known as Turtle Pond (adjacent to Baie Laraie; 4° 17 '12.9" S, 55° 43' 49.1" E), a variety of environmental conditions were assessed. Water temperature was measured using HOBO Pendant® Temperature/Light 64K Data Loggers (Model UA-002-64, ONSET, USA). Loggers were also deployed at depth intervals of 1 m to calculate the light attenuation coefficient (K_d) and therefore turbidity. Water

samples (*n* = 12 per site) were taken over two weeks in April 2018 in order to assess nutrient loading, including dissolved organic carbon (DOC), particulate organic carbon (POC), total dissolved nitrogen (TDN), and total dissolved phosphorus (TDP). Water samples from both habitats were taken in triplicate (1.5 L each) at high tide. Water samples were syringe-filtered through pre-combusted (4 h at 450°C) 0.7µm GF/F filters to separate dissolved and particulate fractions. Dissolved organic and inorganic carbon and total dissolved nitrogen were analysed on a Formacs TOC Auto-analyser (Skalar). Particulate carbon was analysed by Primacs TOC analyser. Dissolved phosphorus was measured following the colorimetric molybdenum blue method (Murphy & Riley, 1962). To assess benthic community structure, 30 m continuous line intercept transects were conducted using SCUBA (*n* = 3 per site, per year), with all benthic video data recorded using cameras (GoPro Hero series 2015-2017). Analysis of video data involved calculating percentage cover of the following benthos: live coral (HC), dead coral (DC), rock (RC), rubble (RB), sand (SD), algae (AG). Corals were identified to genus level (and species level when possible).

3.2.2. Coral collection

In April 2017, ten small colonies of *Porites lutea* were collected from both the fore-reef environment of Home Reef, and the mangrove environment of Turtle Pond. Five of these were reciprocally translocated to the other environment, while the remaining half were transplanted back in their native habitat (back-transplanted controls), following a fully factorial design. To determine the time scale at which coral-associated microbial community change occurs following transplantation to a new environment, coral tissue samples were taken within an hour of collection from the site (T0), and then at 6 hours (T6), 20 hours (T20), and 44 hours (T44) after transplantation. In order to compare the microbiome composition of cross-transplanted corals with back-transplanted conspecifics, the same

tagged corals in both reef and mangrove habitats were revisited and sampled one year after transplantation in April 2018 (*n* = 9 found at Home Reef, *n* = 10 at Turtle Pond).

Small coral tissue samples (< 2cm) were immediately preserved in 2 ml RNAlater (Ambion, Inc.), stored at 4°C for 24h, then transferred to -20°C for shipping and storage. Fragments were transported to University of Essex, UK, where samples in RNAlater were stored at -20°C for subsequent multi-marker amplicon sequencing.

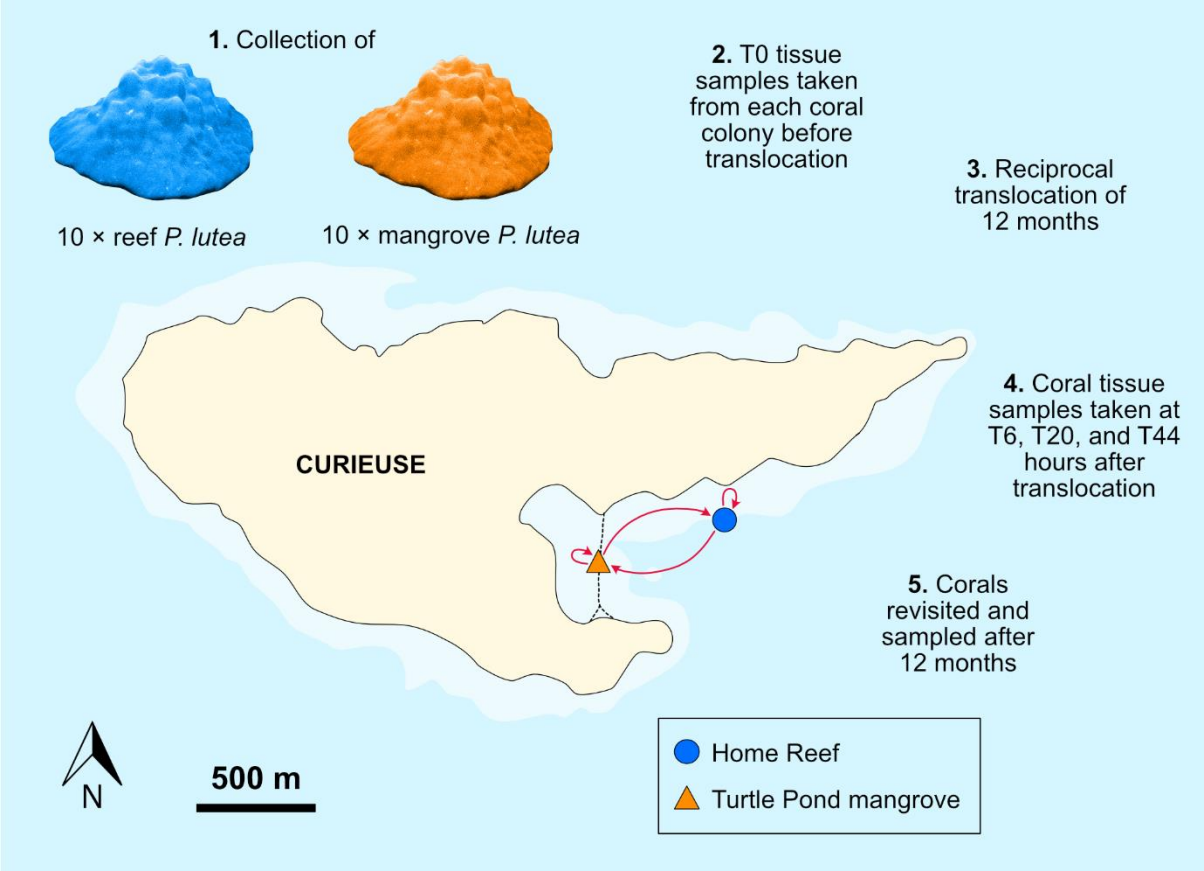


Figure 3.1 Schematic of reciprocal translocation experiment. Ten colonies of *Porites lutea* from each site were collected, and tissue samples taken for DNA analysis, before reciprocal translocation. Red arrows show back-transplantation and cross-transplantation of coral colonies between fore-reef and mangrove sites within the Curieuse Marine National Park. Mangrove site adjacent to the broken seawall within Turtle Pond (shown by a dotted black line). GPS locations for fore-reef site 'Home Reef': 4° 17' 05.1" S, 55° 44' 07.6" E, Mangrove site 'Turtle Pond': 4° 17' 12.9" S, 55° 43' 49.1" E.

3.2.3. DNA extraction

The DNeasy Power Biofilm kit (Qiagen) was used to extract genomic DNA from corals, with minor changes to the protocol. Between 0.05 and 0.1 g of material (coral tissue intact with skeleton) was placed into 2 ml bead-beating tubes containing the manufacturers mix of 0.1, 0.5 and 2.4 mm glass and ceramic beads. Chemical lysis buffers BF1 (350 µl) and BF2 (100 µl) were added and tubes incubated at 65°C for 15 minutes before bead-beating at 6400 rpm for 30s in a Precellys 24 (Bertin Technologies). Tubes were centrifuged at 13000 × *g* for 1 minute before transferring 330 µl of the resulting supernatant to a clean 2 ml microcentrifuge tube and adding 200 µl of patented Inhibitor Removal Solution (BF3). Tubes were left on ice for 1 hour to precipitate non-DNA organic and inorganic material including humic acid, cell debris, polyphenolics, polysaccharides and proteins. Avoiding the pellet, 400 µl of supernatant was added to 900 µl BF4 before proceeding with column-based clean up as per the manufacturer's instructions. Clean DNA was eluted in 100 µl 10 mM Tris buffer before storing frozen at -20°C.

Extracts were viewed on a 1% agarose gel stained with 0.5 µl SybrSafe dye (Thermo Fisher Scientific) loaded with 5 µl DNA extract and 1 µl loading dye. The gel was run by electrophoresis at 90 V for 40 minutes. DNA extracts were also assessed for concentration and purity by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

3.2.4. Quantitative real-time PCR (qPCR)

Quantitative real-time polymerase chain reactions (qPCRs) were used to enumerate coral-associated microbiota. Bacterial and archaeal 16S ribosomal RNA genes were amplified, in addition to the Symbiodiniaceae nuclear ribosomal internal transcribed spacer region between the 5.8S and 28S genes (commonly referred to as ITS2).

To ensure standards used for qPCR were relevant to the samples run on the same plate, standards were made from purified PCR products from the same set of coral DNA extracts. A small subset of genomic DNA samples were amplified (using primers in Table 3.1, and 35 cycles of the conditions detailed in Table 3.2), checked for the expected product size by agarose gel electrophoresis, cleaned using GenElute PCR Clean-Up Kit (Sigma Aldrich), and quantified at a 1/20 dilution using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) following manufacturer's instructions using a NanoDrop 3300 Fluorospectrometer. The number of DNA copies present in these standards was calculated as per McKew and Smith (2015).

All qPCR assays were conducted on a CFX384 Touch C1000 Thermal Cycler (Bio-Rad) using SYBR-Green fluorophore. Each reaction was performed in a final volume of 10 µl containing: 5 µl of SensiFAST SYBR No-ROX (Bioline) mastermix reagent, 0.2 µl of each 10 µM primer (Table 3.1), 0.6 µl 1% BSA, 3 µl H_2O and 1 µl of template DNA. Cycling conditions were: 1 cycle of initial denaturation at 95°C for 3 min; 40 cycles of amplification consisting of 95°C for 5 s and 60°C for 30 s, then a final denaturation of 95°C for 5 s, followed by a final cycle of temperature ramping from 65°C to 95°C at 0.5°C per 5 s increment, for melting temperature curve analysis. Melting curve analyses ensured the specificity of the amplifications. A standard curve was created from analysis of the aforementioned PicoGreen-quantified samples 10-fold serially diluted down to DNA concentrations equating to single-digit abundances of the target gene (from DNA concentrations of ~10^7 down to ~10^1 copies of the target gene). Standard curves yielded high efficiencies for all gene regions (bacterial 16S rRNA: 112%, $R^2 = 0.99$, Symbiodiniaceae ITS2: 91%, $R^2 > 0.99$, archaeal 16S rRNA: 71%, $R^2 = 0.99$). Samples were analysed in technical triplicates and averaged when the standard deviation (SD) of the quantitation cycle (Cq) was less than 2. If Cq SD > 2, the technical outlier was removed

before averaging for the biological sample. The resultant number of copies of each gene region per sample were normalised per g of coral tissue that was used for DNA extraction.

3.2.5. Amplicon sequencing library preparation

Taxa specific loci were amplified using primers from Table 3.1 with the addition of a MiSeq overhang sequence (underlined) e.g. 784F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGATTAGATACCCTGGTA,

1061R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACACRRCACGAGCTGACGAC. Reaction mixtures of 25 µl were prepared with 12.5 µl 2x AppTaq RedMix (Appleton Woods), 0.5 µl of forward primer, 0.5 µl of reverse primer (Table 3.1), 1.5 µl 1% BSA, 9 µl H_2O and 1 µl template DNA. Amplification conditions for the bacterial 16S rRNA and Symbiodiniaceae ITS2 PCRs are detailed in Table 3.2 (but reduced to 27 cycles to allow for subsequent indexing). Archaeal 16S rRNA was not included in the final MiSeq library due to low concentrations of DNA even after amplification. The PCR products were separated by electrophoresis at 90 V for 45 minutes on 1.2% agarose gel stained with SybrSafe, and visualised using a UV transilluminator, for confirmation of the correct sized product.

The PCR products were subsequently cleaned with Bioline JetSeq Clean solid phase reversible immobilisation (SPRI) beads (Scientific Laboratory Supplies), indexed over 8 PCR cycles with Nextera XT indexes (Illumina), and cleaned again with JetSeq Clean SPRI beads, following the Illumina 16S MiSeq manual. Each amplicon was quantified in triplicate, using PicoGreen dye (Quant-iT PicoGreen dsDNA Assay Kit, ThermoFisher Scientific), in 384-well plate format, on a plate reader (FLUOstar Omega Microplate Reader, BMG LabTech), before being pooled in equimolar ratios. Resulting gene libraries were pooled at a ratio of 3:1, 16S rRNA gene: ITS2 rRNA region, respectively, and cleaned using GenElute PCR Clean-Up Kit (Sigma Aldrich) to ensure no carry-over of magnetic beads. Sequencing

was performed at 6 pM concentration with 17% phiX control, on the Illumina MiSeq platform, using a 600-cycle MiSeq Reagent Kit v3 (Illumina) to yield 2 × 300 bp overlapping paired-end reads. Negative mock DNA extractions and negative PCR controls were sequenced alongside samples to check for contamination. The resulting cluster density was 371K/mm^2.

Table 3.1. Primers used for qPCR

Target taxa	Primer name	Primer sequence	Amplicon size (bp)	Reference
Bacteria	784F 1061R	AGGATTAGATACCCTGGTA CRRCACGAGCTGACGAC	~277	Andersson *et al.* (2008)
Archaea	SSU1ArF SSU520R	TCCGGTTGATCCYGCBRG GCTACGRRYGYTTTARRC	~519	Bahram *et al.* (2019)
Symbiodiniaceae	Sym_Var_5.8S2 Sym_Var_Rev	GAATTGCAGAACTCCGTGAACC CGGGTTCWCTTGTYTGACTTCATGC	~300	Hume *et al.* (2018)

Table 3.2. Cycling conditions for PCR amplification targeting different microbial taxa.

Target taxa	Primer set	Cycling conditions					
		Initial denaturation	Denaturation	Annealing	Extension	Final extension	Final hold
Bacteria	784F / 1061R	95˚C for 3 min	35 cycles 95˚C for 15 sec	 55˚C for 15 sec	 72˚C for 30 sec	72˚C for 7 min	4˚C ∞
Archaea	SSU1ArF / SSU520R	95˚C for 3 min	35 cycles 95˚C for 30 sec	 55˚C for 30 sec	 72˚C for 30 sec	72˚C for 10 min	4˚C ∞
Zooxanthellae	Sym_Var_5.8S2 / Sym_Var_Rev	95˚C for 3 min	35 cycles 95˚C for 15 sec	 56˚C for 15 sec	 72˚C for 30 sec	72˚C for 5 min	4˚C ∞

Reaction mixtures of 25 µl were prepared with 12.5 µl 2x AppTaq RedMix (Appleton Woods), 1 µl of 10µM forward primer, 1 µl of 10µM reverse primer, 1.5 µl 1% BSA, 8 µl H_2O and 1 µl template DNA.
Fungal primers fITS7 / ITS4 (Ihrmark *et al*., 2012) were trialled, but found to also amplify Symbiodiniaceae, so were removed from further analysis and not included in MiSeq library preparation.

3.2.6. Bioinformatics

The bacterial amplicon library was processed following (Dumbrell *et al.*, 2017). Briefly, sequence reads were trimmed to 200 bp, before being quality trimmed using Sickle (Joshi & Fass, 2011), error corrected in SPAdes (Bankevich *et al.*, 2012) using the BayesHammer algorithm (Nikolenko *et al.*, 2013), and pair-end aligned with a minimum overlap of 15 bp with PEAR (Zhang *et al.*, 2014) within PANDASeq (Masella *et al.*, 2012). Any pair-end aligned sequences shorter than 180 bp were removed. The quality-filtered, error-corrected, and pair-end aligned sequences were then de-replicated, sorted by their abundance, and OTU centroids picked using VSEARCH (Rognes *et al.*, 2016) at the 97% similarity level. All singleton OTUs were removed. Chimeric sequences were removed using reference-based chimera checking with UCHIME (Edgar *et al.*, 2011). Bacterial sequences were assigned to taxa using a naïve Bayesian rRNA classifier, with a 60% bootstrap confidence threshold (RDP Classifier; Wang *et al.*, 2007).

The ITS2 amplicon library was processed remotely using the SymPortal analytical framework (Hume *et al.*, 2019). Demultiplexed, paired forward and reverse sequences (fastq.gz output files from Illumina MiSeq) were submitted to SymPortal.org for remote quality control (Mothur 1.39.5; Schloss *et al.*, 2009, and BLAST + ; Camacho *et al.*, 2009) and minimum entropy decomposition (Eren *et al.*, 2015), before resolving putative Symbiodiniaceae taxa (ITS2-type profiles) by defining intragenomic ITS2 sequence variants (DIVs).

3.2.7. Microbial community analysis

Analyses were carried out using the `phyloseq` package within `R` (McMurdie & Holmes, 2013). Any sequences classified as belonging to domains other than Bacteria, were filtered from the dataset before rarefaction. Samples were rarefied (sub-sampled) to 5000

sequences to attain a depth sufficient to capture the diversity of most samples, and any samples with a coverage lower than 5000 sequences were excluded from further analyses.

Alpha diversity metrics (OTU richness, Pielou's evenness, and Shannon-Wiener diversity) of coral-associated bacterial communities were calculated for each coral sample using `*estimate_richness*` function in `phyloseq`. Since such diversity metrics are directly correlated, a multivariate analysis of variance (MANOVA) was used to compare coral-associated bacterial assemblages based on the source and sampled habitat of the coral host, as well as the effect of time following translocation.

Bacterial community composition at the family level was compared visually using stacked bar graphs plotted using `ggplot2` in R (Wickham, 2009). Mean percentage abundances of phylogenetically annotated 16S rRNA gene sequences from replicate samples were plotted for reciprocally transplanted *Porites lutea* colonies, before, then 20 hours, 44 hours, and one year after, translocation.

Differences in bacterial community composition between coral and seawater samples, and between corals from different habitats (source habitat: mangrove vs reef; and sampled habitat: mangrove vs reef) were visualised using non-metric MultiDimensional Scaling (nMDS), based on Bray–Curtis dissimilarity distances. Differences in coral-associated bacterial and Symbiodiniaceae communities between reef and mangrove source habitats, and sampled (destination) habitats, were compared over time using permutational multivariate analysis of variance (PERMANOVA) with the `*adonis*` function from the R package `vegan`. Each three-way PERMANOVA was run with 999 permutations, and was based on Bray–Curtis dissimilarity distances.

To test for differentially abundant bacterial taxa between source and sampled habitats, as well as any interaction effects, a generalised linear model (GLM) with negative binomial distribution assumption was fitted, using the `DESeq2` package (version 1.24.0) in `R` (Love

et al., 2014). Random effects are not supported in `DESeq2` so coral colony was not included in the model. Although it is often recommended that `DESeq2` be used with raw, unrarefied count data (McMurdie & Holmes, 2014), due to relatively large differences in group library sizes, rarefied data were used here, in conjunction with the `DESeq2` default Benjamini-Hochberg correction for adjusted p-values, to ensure a low false discovery rate (FDR) (Weiss *et al.*, 2017).

Symbiodiniaceae ITS2 type profiles (the taxonomic unit of SymPortal) are genotypes representative of putative taxa (Hume *et al.*, 2019). Pie charts were plotted to track the ITS2 type profile of each coral colony over time following transplantation. Symbiodiniaceae community composition was also visualised as mean percentage of sequence variants (DIVs) using stacked bar graphs (`ggplot2`; Wickham, 2009).

3.3. Results

3.3.1. Abiotic conditions of mangrove vs. reef habitat

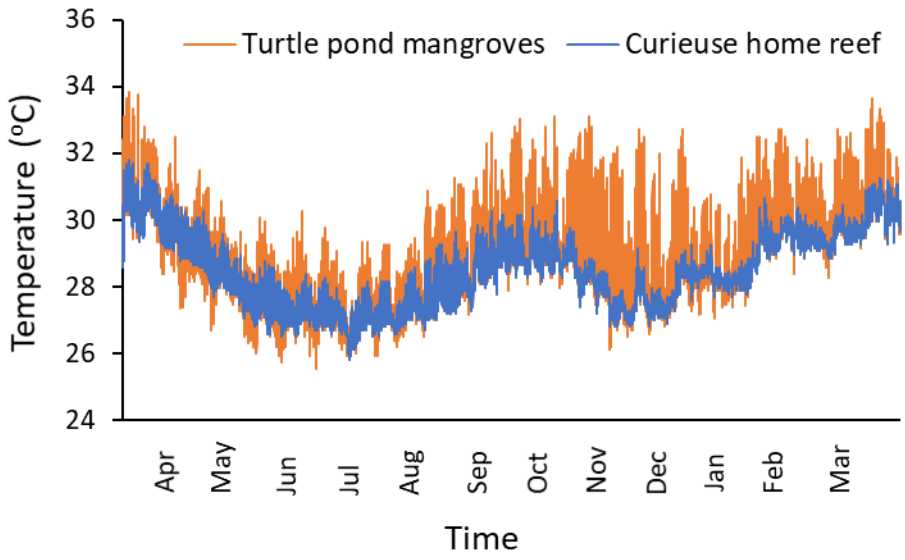


Figure 3.2. One year time series of sea temperature for Curieuse Home Reef (blue) and Turtle Pond mangrove (orange), Seychelles.

Thermal conditions of the mangrove, Turtle Pond, were more extreme than at Home Reef (Fig. 3.2). Turtle Pond mangrove experiences water temperatures as high as 33.9°C and daily fluctuations of up to 5.3°C, compared with Home Reef maximum temperature of 31.8°C and maximum daily range of 2.3°C (measured from April 2017 – April 2018). However, nutrient loading was not noticeably different between the two habitats, though this was based on limited water sampling in only one month (April) of each year (Fig. 3.3).

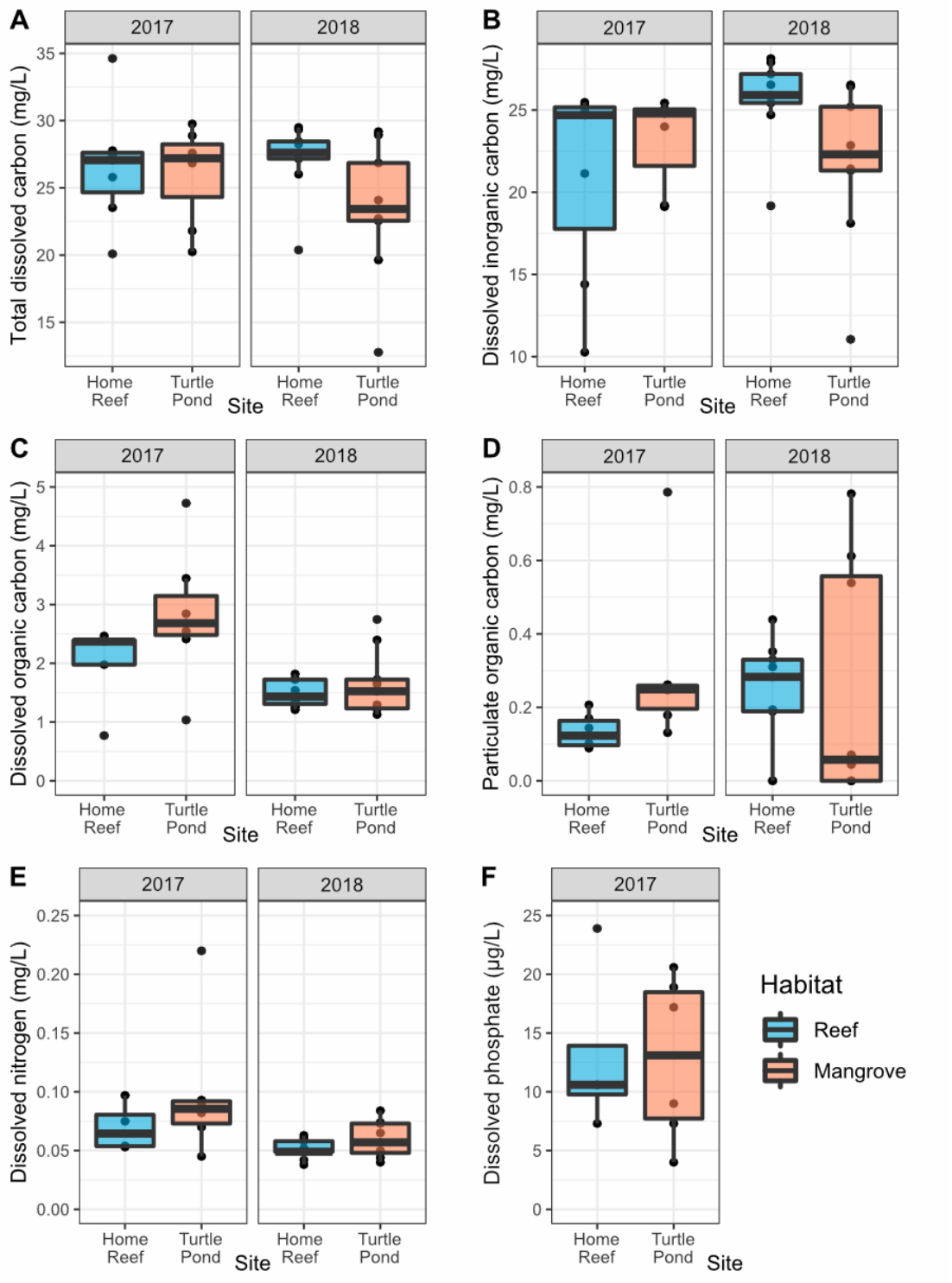


Figure 3.3. Nutrient loading of water from Curieuse Home Reef (blue) and Turtle Pond mangrove site (orange) in April 2017 and 2018. **A)** Total dissolved carbon; **B)** Dissolved inorganic carbon; **C)** Dissolved organic carbon; **D)** Particulate organic carbon; **E)** Total dissolved nitrogen; **F)** Dissolved phosphate. Boxplots show median and interquartile range. Points show raw values (n = 7 per habitat in 2017 and n = 9 per habitat in 2018).

3.3.2. Benthic community composition

Coral cover data from video line intercept transects (LITs) showed differences in total substrate cover of hard coral, as well as genus composition of live hard corals (Fig. 3.4). The main difference in coral assemblages between the reef and mangrove habitats was little dead coral cover in the mangrove (< 1% in 2017, and 0% in 2018) compared to the reef (6% in 2017, and 11% in 2018). Of the live hard coral cover, the mangrove was dominated by branching taxa such as *Acropora* spp. (48% of live coral in 2017, and 75% in 2018), while the reef was dominated by massive morphologies e.g. *Porites lutea* and *Pavona clavus* (84% of live coral in 2017, and 80% in 2018).

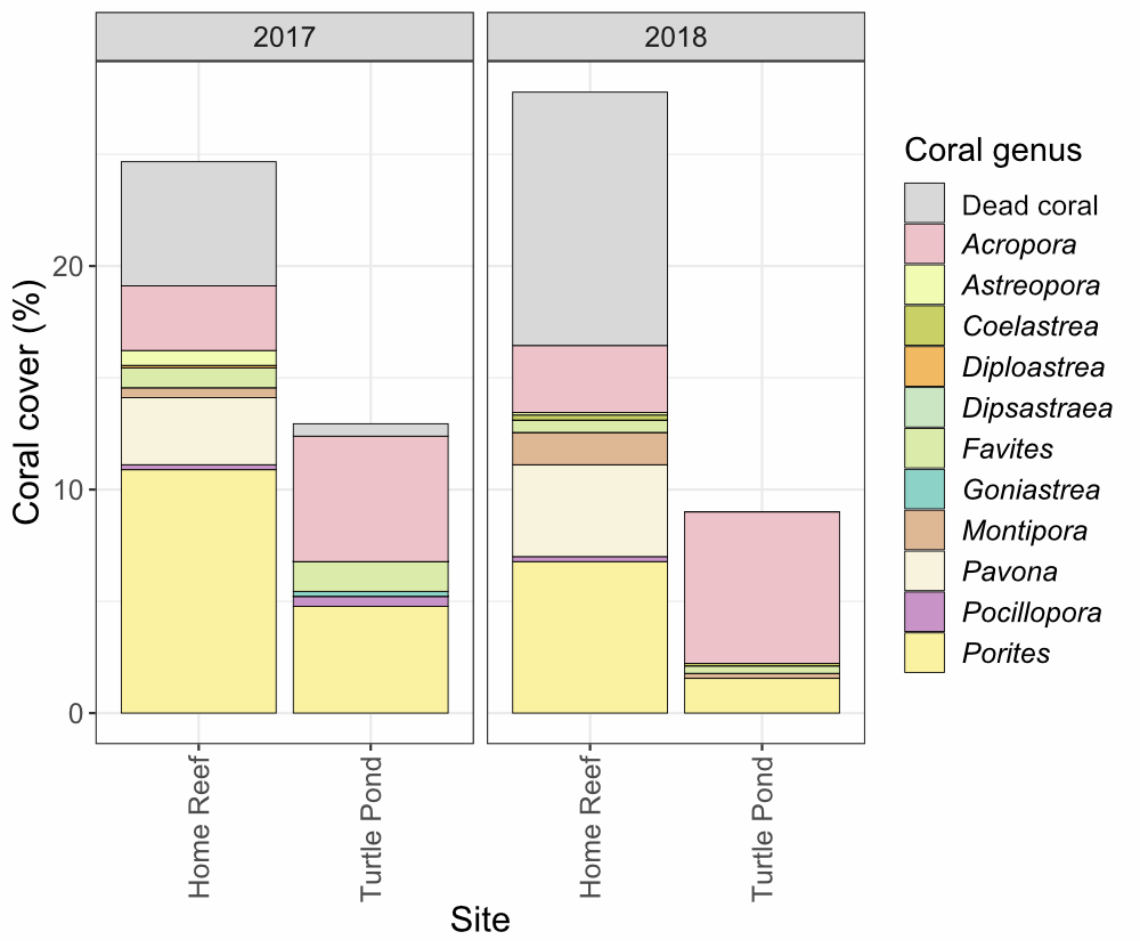


Figure 3.4. Average percentage hard coral cover (%) measured along 30 m transects (*n* = 3) at Home Reef and Turtle Pond Mangrove in 2017 and 2018. Hard coral cover is coloured at the taxonomic level of genus, except for dead coral, shown in grey.

3.3.3. Coral transplant survival

To address whether there had been trade-offs for translocation to a new environment, coral transplants' ID tags were checked, and survival noted, one year after transplantation. All of the coral colonies which could be found had survived one year of translocation, though some mangrove to reef transplants exhibited signs of bleaching (Table 3.3).

Table 3.3. Survival summary of *Porites lutea* transplants around Curieuse Island, Seychelles, one year after translocation.

Translocation	*P. lutea* survival	*n*
Reef to reef	100%	5
Reef to mangrove	100%	5
Mangrove to mangrove	100%	5
Mangrove to reef	100% *	4 †

*Two of the four recovered mangrove to reef transplants exhibited bleaching of half the colony.
† One coral colony was missing after one year on the reef.

3.3.4. Coral-associated microbial abundance is highly variable

Coral-associated 16S rRNA amplicon abundance (a proxy for bacterial abundance) was extremely variable in *Porites lutea* from both reef and mangrove habitats, spanning five orders of magnitude (reef-sampled *P. lutea*: $4.13 \times 10^6 – 1.44 \times 10^{11}$, vs. mangrove-sampled *P. lutea:* $5.16 \times 10^6 – 1.61 \times 10^{12}$ 16S rRNA gene copies g^{-1} coral tissue). While there appears to be a slight increase in bacterial loading of cross-transplanted corals within 6 hours, followed by a decrease over time, coral-associated bacterial abundance was too variable to draw conclusions on the impact of translocation (Fig. 3.6). Meanwhile, bacterial loading of seawater from each habitat was less variable (reef water: $1.70 \times 10^7 – 5.54 \times 10^8$, vs. mangrove water: $3.36 \times 10^7 – 9.83 \times 10^8$ rRNA copies L^{-1} seawater), and generally slightly higher in the mangrove (Fig. 3.5). Symbiodiniaceae abundance ranged from 2.25×10^8 to 1.79×10^{10} and 5.80×10^7 to 1.46×10^{10} g^{-1} in reef and mangrove sampled corals, respectively (Fig. 3.6). Symbiodiniaceae loading in surrounding reef (4.00×10^3 to 8.61×10^4 L^{-1}) and mangrove (2.01×10^3 to 2.15×10^4 L^{-1}) seawater was substantially lower (Fig. 3.5).

Abundance of coral-associated archaea was several orders of magnitude lower than bacteria with corals sampled at the reef hosting 2.22×10^4 to 6.14×10^6 g^{-1} and corals sampled at the mangrove hosting 1.13×10^4 to 4.56×10^7 archaeal 16S rRNA gene copies g^{-1} coral tissue (Fig. 3.6). Archaeal loading of seawater ranged from 3.95×10^4 to 6.01×10^5 L^{-1} on the reef, and from 6.30×10^3 to 1.15×10^6 L^{-1} in the mangrove (Fig. 3.5). Due to the high intra- and inter-colony variability in microbial loading, there was no effect of source habitat, nor destination habitat over time on bacterial, Symbiodiniaceae, nor archaeal loading of corals ($P > 0.05$ for all repeated measures ANOVAs of $\log_{10}$-transformed gene copies; Fig. 3.6).

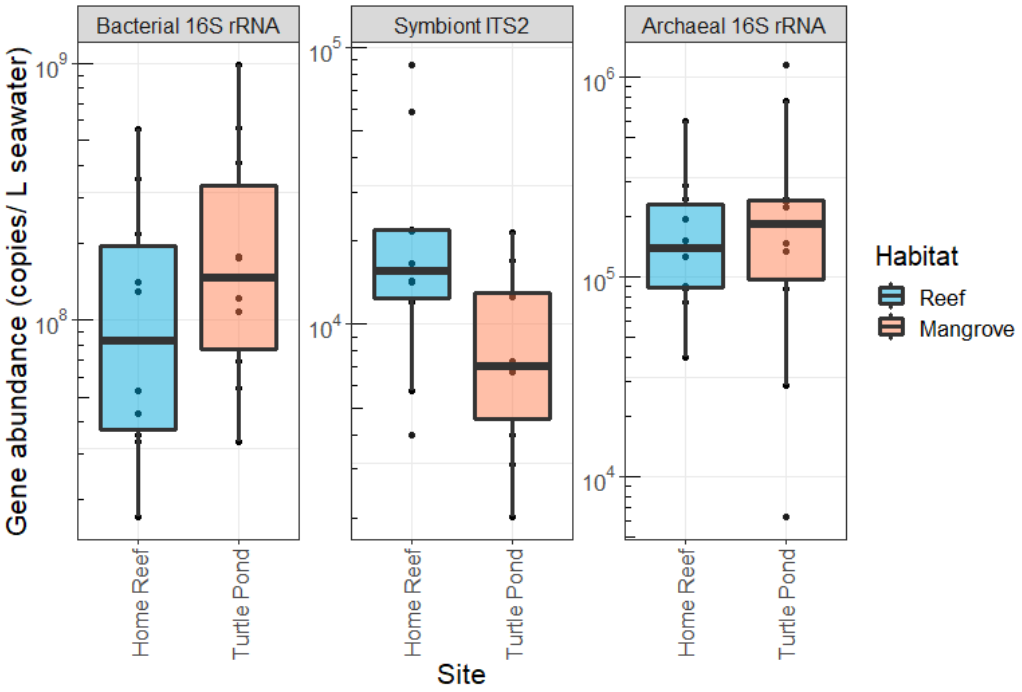


Figure 3.5. Microbial loading of seawater (bacterial 16S rRNA, Symbiodiniaceae ITS2, archaeal 16S rRNA gene copies per litre) from reef (Home Reef) and mangrove-influenced (Turtle Pond) habitat. Boxplots represent median and interquartile range (n = 10 samples per habitat; blue = reef; orange = mangrove water), plotted on a logarithmic scale. Note that the different taxa are plotted on separate scales.

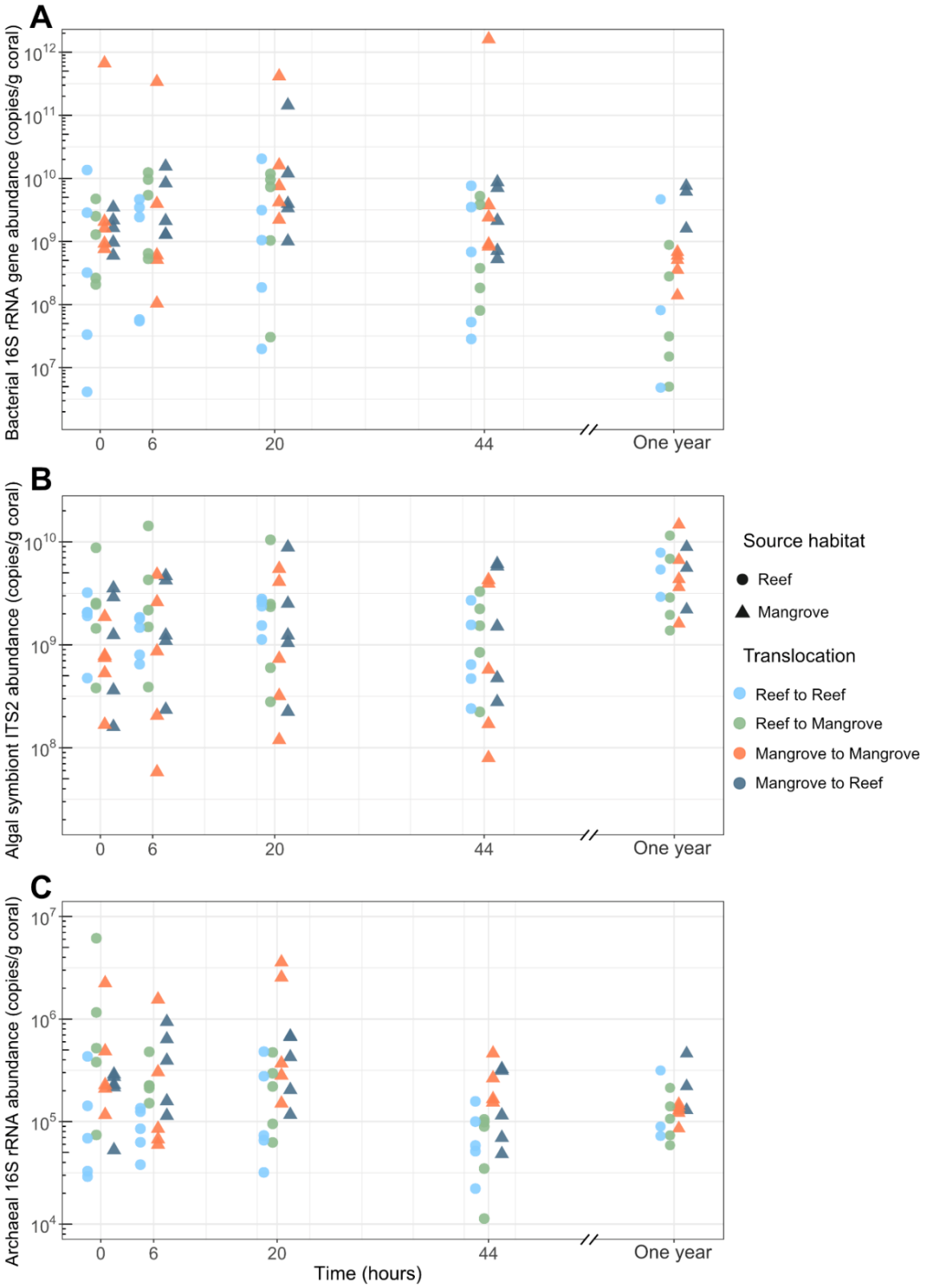


Figure 3.6. Microbial loading of *Porites lutea* before translocation (T0), 6 hours (T6), 20 hours (T20), 44 hours (T44), and one year after translocation, from reef to reef (light blue), reef to mangrove (green), mangrove to mangrove (orange), and mangrove to reef (dark blue). Corals sampled at T0 were still in their source habitat. **A)** Bacterial 16S rRNA **B)** Algal symbiont ITS2 **C)** Archaeal 16S rRNA. Values are gene copies per g wet weight coral tissue plotted on a logarithmic scale (n = 5 per translocation, per time point).

3.3.5. Changes in bacterial community composition

Amplicon sequencing of bacterial 16S rRNA gene amplicons produced sequences that clustered into 10563 distinct OTUs from 93 samples. After filtering of non-target taxa (archaea, chloroplast, mitochondria sequences) and rarefaction to a depth of 5000 sequences per sample, there were 7108 taxa from 85 samples.

3.3.5.1. Bacterial communities of seawater and coral are distinct

Seawater samples were dominated by unclassified Bacteroidetes (averaging 19% relative abundance from the reef, and 18% from the mangrove) and bacteria from the Flavobacteriaceae family (averaging 16% from the reef, and 14% from the mangrove). The main difference in bacterial community composition of mangrove and reef water was due to a higher average relative abundance of Rhodobacteraceae in the mangrove-influenced seawater (19%) compared with the reef-sampled seawater (7%). Vibrionaceae were similarly abundant in both reef and mangrove water, averaging 2.8% abundance in seawater from the reef and 2% in the mangrove seawater (ranging from 0% to 7% across all samples). Other bacterial families which were present at average relative abundances greater than 1% included Pelagibacteraceae (SAR11; 6% reef, 2% mangrove), Litoricolaceae (3% reef, 4% mangrove), Saprospiraceae (3% reef, 1% mangrove), Alteromonadaceae (1% reef, 2% mangrove), Cryomorphaceae (<1% reef, 1% mangrove), and Oceanospirillaceae (<1% reef, 1% mangrove). Of the 3873 OTUs identified from water samples, only 1127 (~29%) were shared with at least one of the coral samples. The distinct differences in bacterial community composition between coral and seawater samples were clearly illustrated by nMDS (Fig. 3.7). To focus on differences between the coral-associated bacterial communities of translocated *P. lutea*, seawater samples were excluded from subsequent analyses.

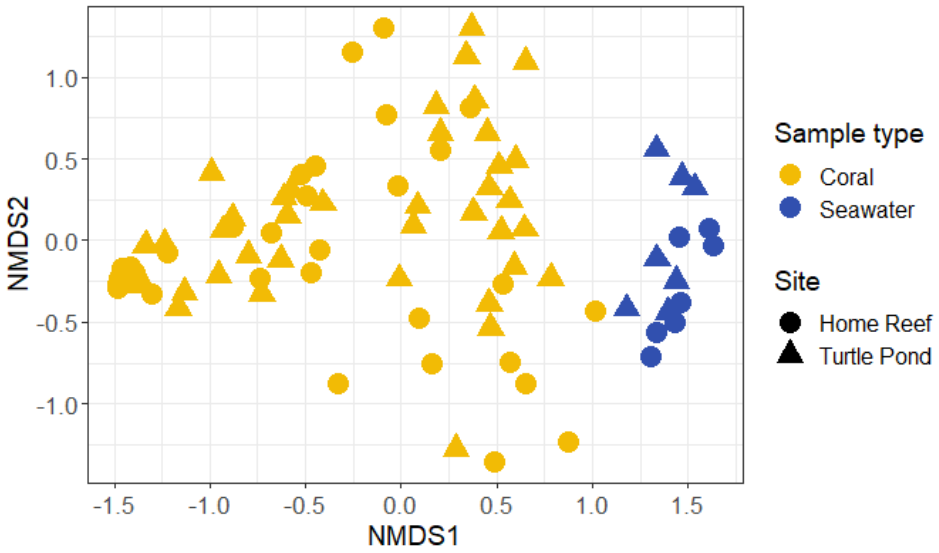


Figure 3.7. Non-metric MultiDimensional Scaling (nMDS) of bacterial community composition based on Bray-Curtis dissimilarity (2D stress = 0.18). Each symbol represents a sample, symbol colours denote sample type (yellow = *Porites lutea* coral, blue = seawater), symbol shapes denote sampling site (circle = Home Reef, triangle = Turtle Pond mangrove).

3.3.5.2. Coral-associated bacterial diversity

The bacterial OTU richness and diversity associated with *P. lutea* living in a mangrove habitat could not be easily distinguished from the bacterial richness and diversity hosted by corals living in a reef habitat. When all alpha diversity metrics (OTU richness, evenness, and diversity) were considered, there was an effect of sampled (destination) habitat, time, and their interaction (i.e. the effect of sampled habitat changed over time; Table 3.4). Generally, OTU richness and evenness of coral-associated bacteria decreased over time following translocation (Fig. 3.8). Before translocation, reef and mangrove corals exhibited no difference in the average bacterial OTU richness or diversity hosted, however there was generally more variation in bacterial richness and diversity between reef corals, than between mangrove corals Fig. 3.8). Within time points, none of the translocations were significantly different in terms of bacterial richness or diversity from one another ($P > 0.05$). Between time points however, mangrove to reef transplanted corals differed in richness, evenness and Shannon diversity between sampling before translocation, and one year after ($P < 0.05$). However, this was not likely due to translocation, as mangrove to mangrove

back-transplanted corals also exhibited a significant decrease in bacterial evenness and therefore diversity between before, and one year after back-transplantation ($P < 0.05$).

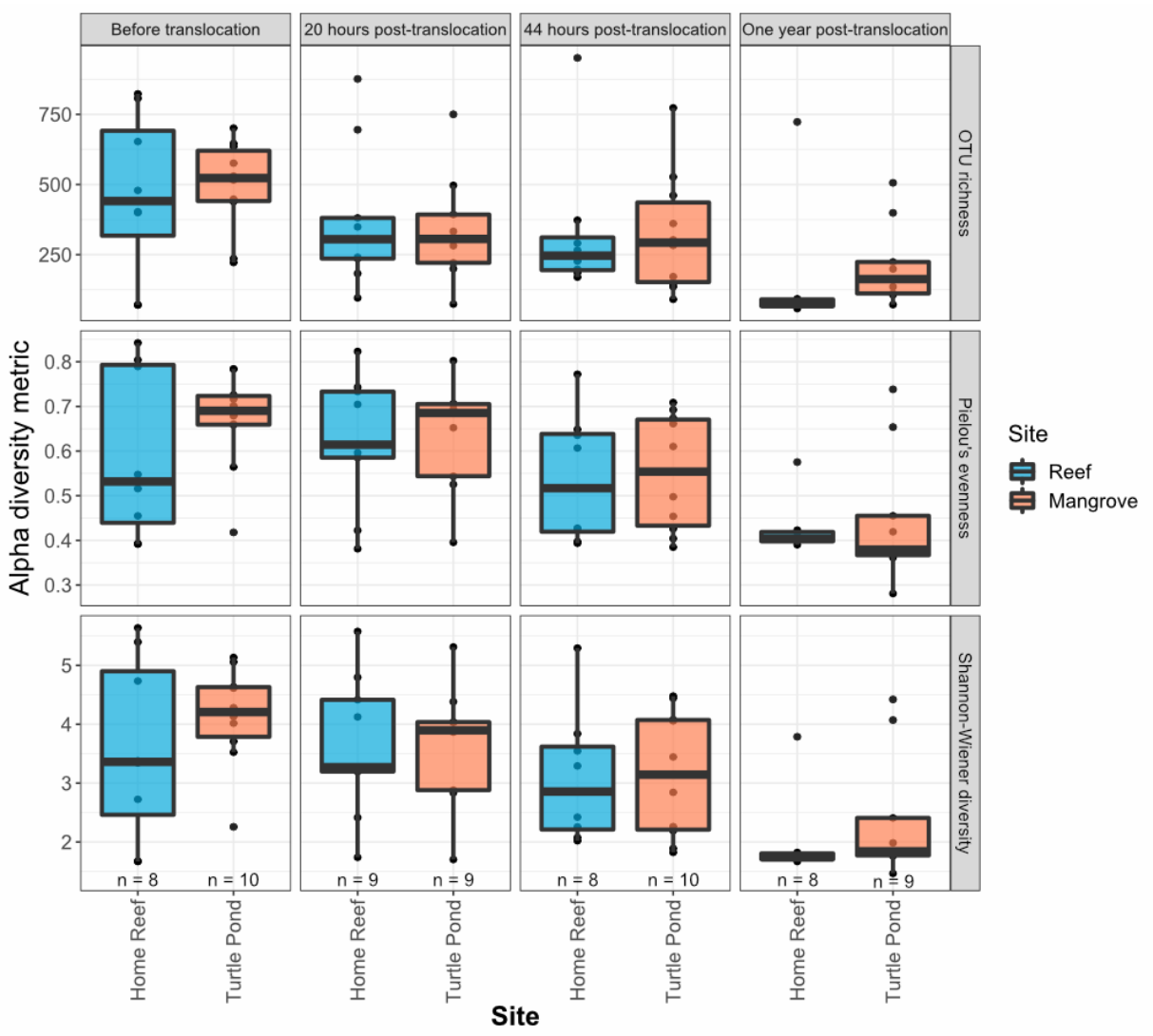


Figure 3.8. Alpha diversity measures (OTU richness, Pielou's evenness, Shannon-Wiener diversity) of bacterial community associated with *Porites lutea* sampled at reef (Home Reef) and mangrove-influenced (Turtle Pond) habitat, before and after translocation (top facets show time point). Values are median and interquartile range, based on counts rarefied to 5000 reads per sample.

Table 3.4. Statistical comparison of coral-associated bacterial diversity metrics between habitats, over time. Data were analysed using a multivariate analysis of variance (MANOVA) i.e., multiple dependent variables (OTU richness, evenness, and diversity) were analysed simultaneously. Diversity indices and subsequent tests were calculated based on counts rarefied to 5000 sequences per sample.

Multivariate Analysis of Variance					
Factor(s)	**Pillai's trace statistic**	***F***	**df**	**df $_{Error}$**	***p*-value**
Source habitat	0.046	0.866	3	54	0.465
Sampled site	0.207	4.709	3	54	**< 0.01**
Time	0.495	3.687	9	168	**< 0.001**
Source × Sampled site	0.015	0.268	3	54	0.848
Source habitat × Time	0.161	1.057	9	168	0.397
Sampled site × Time	0.314	3.415	6	110	**< 0.01**
Source × Sampled site × Time	0.083	0.798	6	110	0.574

Univariate effects				
Factor(s)	**Diversity metric**	***F***	**df**	***p*-value**
Source habitat	OTU richness	0.653	1	0.423
	Pielou's evenness	1.622		0.208
	Shannon-Wiener diversity	1.560		0.217
Sampled site	OTU richness	0.004	1	0.949
	Pielou's evenness	0.256		0.615
	Shannon-Wiener diversity	0.303		0.584
Time	OTU richness	4.560	3	**< 0.01**
	Pielou's evenness	7.755		**< 0.001**
	Shannon-Wiener diversity	7.627		**< 0.001**
Source habitat × Sampled site	OTU richness	0.027	1	0.870
	Pielou's evenness	0.137		0.712
	Shannon-Wiener diversity	0.061		0.805
Source habitat × Time	OTU richness	2.443	3	0.074
	Pielou's evenness	2.078		0.113
	Shannon-Wiener diversity	2.484		0.070
Sampled site × Time	OTU richness	0.084	2	0.920
	Pielou's evenness	0.007		0.993
	Shannon-Wiener diversity	0.080		0.923
Source × Sampled site × Time	OTU richness	0.065	2	0.938
	Pielou's evenness	0.194		0.824
	Shannon-Wiener diversity	0.139		0.870

Statistically significant comparisons shown in bold.

3.3.5.3. Coral-associated bacterial communities are transient and habitat-driven

Following sample rarefaction, a total of 5370 bacterial OTUs were identified as associated with *Porites lutea*. Of the 3436 OTUs associated with *P. lutea* before translocation, only 606 OTUs were shared between any two samples from different habitats. Thus, before translocation, the bacterial community composition associated with *Porites lutea* from mangrove and reef habitat were distinctly different. Non-metric MultiDimensional Scaling (nMDS) ordination illustrated the high dissimilarity in bacterial community composition between corals from different habitats (see 'before translocation' facet of Fig. 3.9). However, within 20 hours of coral translocation, the bacterial community had shifted, such that mangrove and reef sampled corals were no longer distinct (i.e. they were not dissimilar; Fig. 3.9). The same coral colonies sampled one year after translocation showed no differentiation by source habitat nor sampled habitat. However, bacterial communities from reef-sampled corals did tend to cluster together, with the exception of colony D (the blue circle to the far right of the one year post-translocation facet in Fig. 3.9).

Coral-associated bacterial community compositions one year after translocation were significantly different to those of earlier time points, due to a decrease in diversity (Fig. 3.9) caused by a loss of certain taxa. Those most significantly affected by time (DESeq2 analysis) i.e. those probably most highly abundant before translocation were *Shimia* (OTU 12), Oceanospirillaceae (OTU71, OTU162, OTU 241, OTU 178, OTU 188), Flavobacteriaceae *Nonlabens* (OTU110), Rhodobacteraceae (OTU 716), *Meridianimaribacter* (OTU 30), Alteromonadaceae *Salinimonas* (OTU4808).

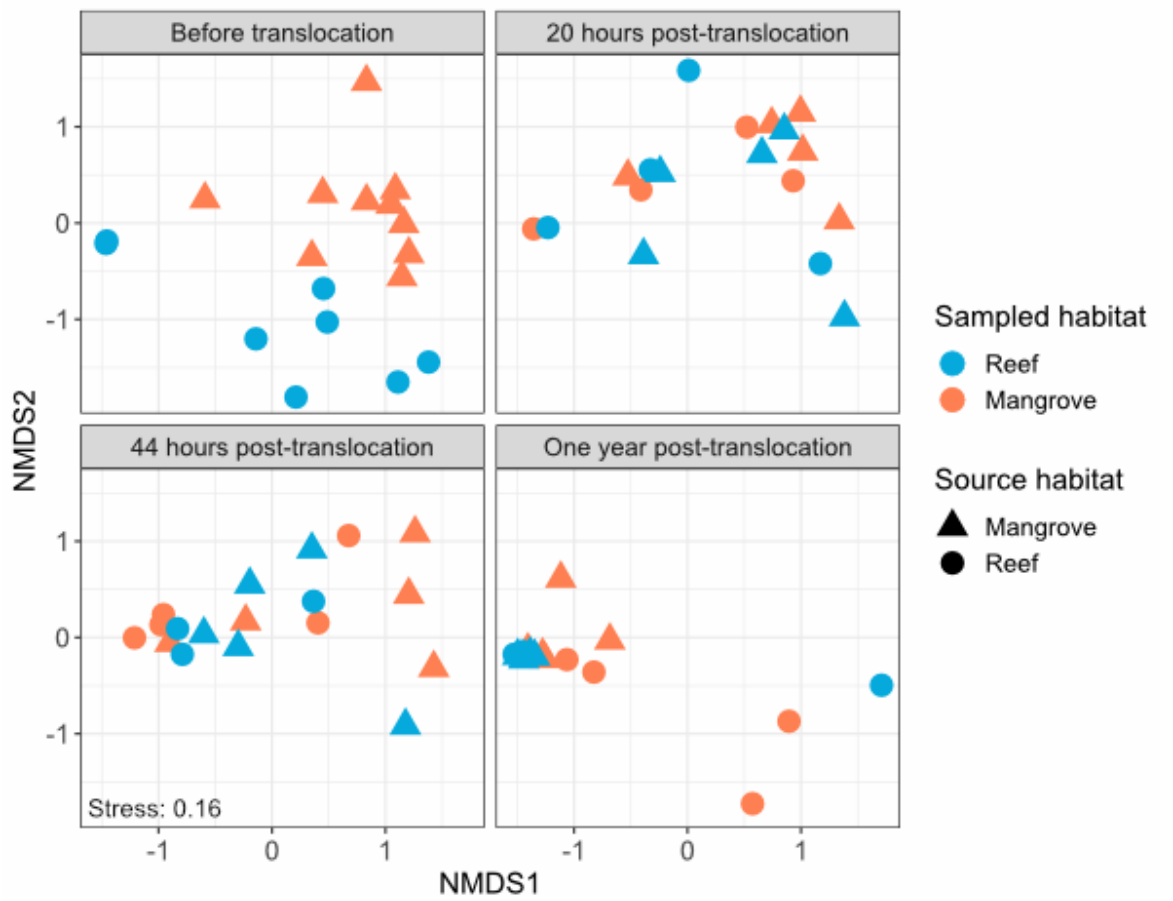


Figure 3.9. Non-metric MultiDimensional Scaling (nMDS) ordination of bacterial community composition based on Bray-Curtis dissimilarity (2D stress = 0.16). Each symbol represents a sample, symbol shapes denote habitat of origin (circles = reef, triangles = mangrove), symbol colours denote habitat of destination (blue = reef, orange = mangrove). Panels show time points in the reciprocal translocation experiment (before translocation, 20 hours post-translocation, 44 hours post-translocation, one year post-translocation).

Table 3.5. Statistical comparison of the composition of *Porites lutea* coral-associated microbiome between habitats and across time after translocation. Data were analysed using a permutational analysis of variance (PERMANOVA) with 999 permutations and based on Bray–Curtis dissimilarity distances.

Three-way PERMANOVA			**Pairwise comparisons by time point**				
Factor	**Model-F**	**P**		**Before**	**T20**	**T44**	**One year**
Source site	2.959	**< 0.05**	Before	-	1.575	1.267	4.569
Sampled site	2.403	**< 0.05**	T20	0.738	-	2.138	8.587
Time	3.630	**< 0.001**	T44	1.000	0.408	-	3.602
Source × Sampled site	0.895	0.421	One year	**< 0.05**	**< 0.01**	0.072	-
Source site × Time	1.393	0.148					
Sampled site × Time	0.809	0.580					
Source × Sampled site × Time	0.480	0.962					

Significant comparisons shown in bold. Pairwise comparisons between time points: upper values are model F-values, lower values are *p*-values.

Throughout the experiment, across both source and sampled habitats, the family Hahellaceae dominated, accounting for at least 12%, and up to 78%, of the average relative abundance. The bacterial families Alteromonadaceae (6%), Campylobacteraceae (2%), Colwelliaceae (2%), Marinifilum (3%), Oceanospirillaceae (6%) and Vibrionaceae (6%) constituted some of the most abundant taxa in corals that originated from the mangrove before translocation, but were either absent or constituted less than 1% of the average relative bacterial abundance in native reef-origin corals (first panel of Fig. 3.10). Rhodobacteraceae also featured in notably higher abundance in native mangrove corals (14%) compared with native reef corals (2%), similar to the pattern seen in seawater from the two habitats.

One year following translocation, both mangrove to reef transplanted corals and back-transplanted mangrove corals lost diversity (in terms of OTU richness; Fig. 3.8, and bacterial

family richness; Fig. 3.10), and became heavily dominated (78%) by the bacterial family Hahellaceae which includes the known coral symbiont *Endozoicomonas*.

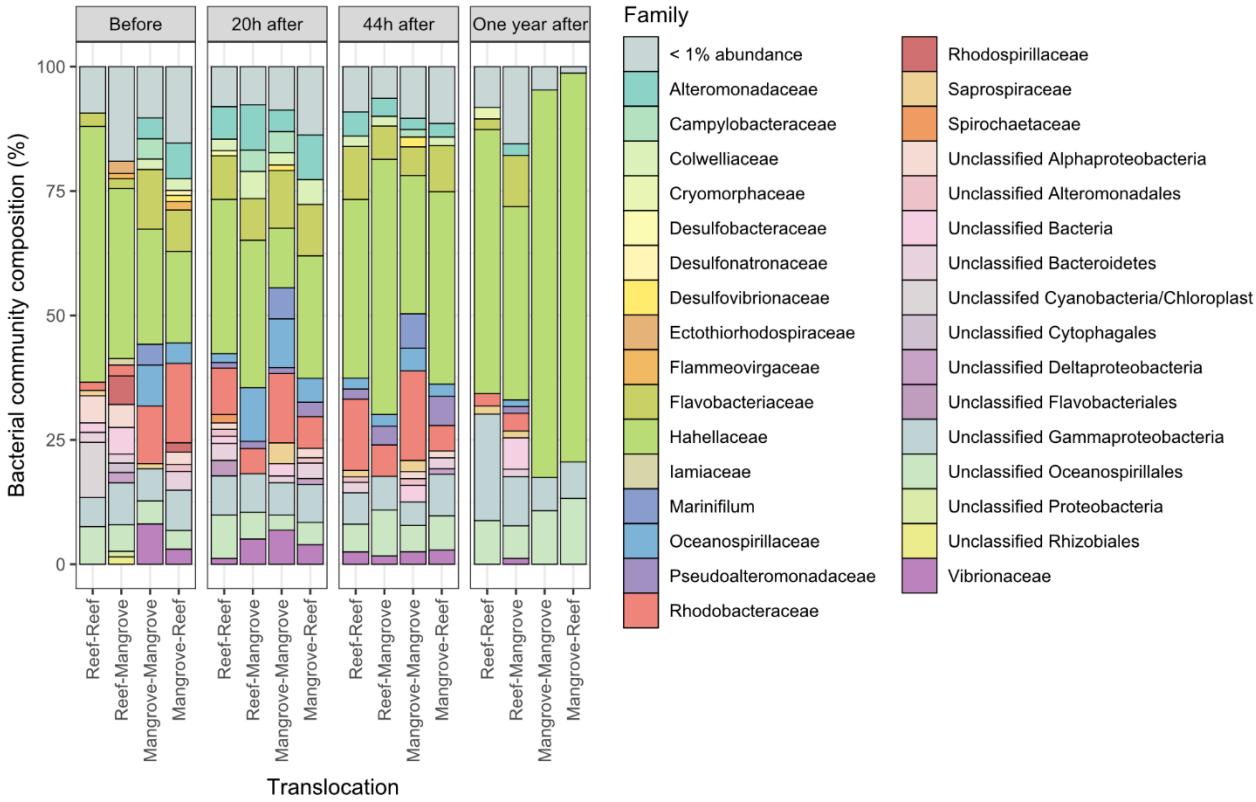


Figure 3.10. Average relative abundance (%) of bacterial families (based on 16S rRNA gene sequences), associated with *Porites lutea,* reciprocally translocated between mangrove and reef habitat. Panels show time points in the reciprocal translocation experiment (before translocation, 20 hours post-translocation, 44 hours post-translocation, one-year post-translocation). Colours represent the most abundant bacterial families (> 1% mean abundance). Remaining taxa are grouped as '< 1% abundance'.

3.3.5.4. Differential abundance of key bacterial taxa

Differential abundance analysis with DESeq2 (Love *et al.*, 2014) highlighted 45 OTUs which were differentially abundant between the sampled sites, Home Reef and Turtle Pond (Fig. 3.11). The most differentially abundant OTUs belonged to the genus *Marinifilum*, which were sometimes 32-fold (log_2 5-fold) more abundant in mangrove-sampled corals, compared with reef-sampled corals.

Mangrove corals cross-transplanted to Home Reef showed a relatively greater abundance of *Pseudomonas* and *Massilia*, but a relatively reduced abundance of *Desulfovibrio*, *Saprospira*, *Marinifilum*, *Arcobacter*, and *Neptuniibacter* (all taxa which were revealed to be more associated with Turtle Pond mangrove).

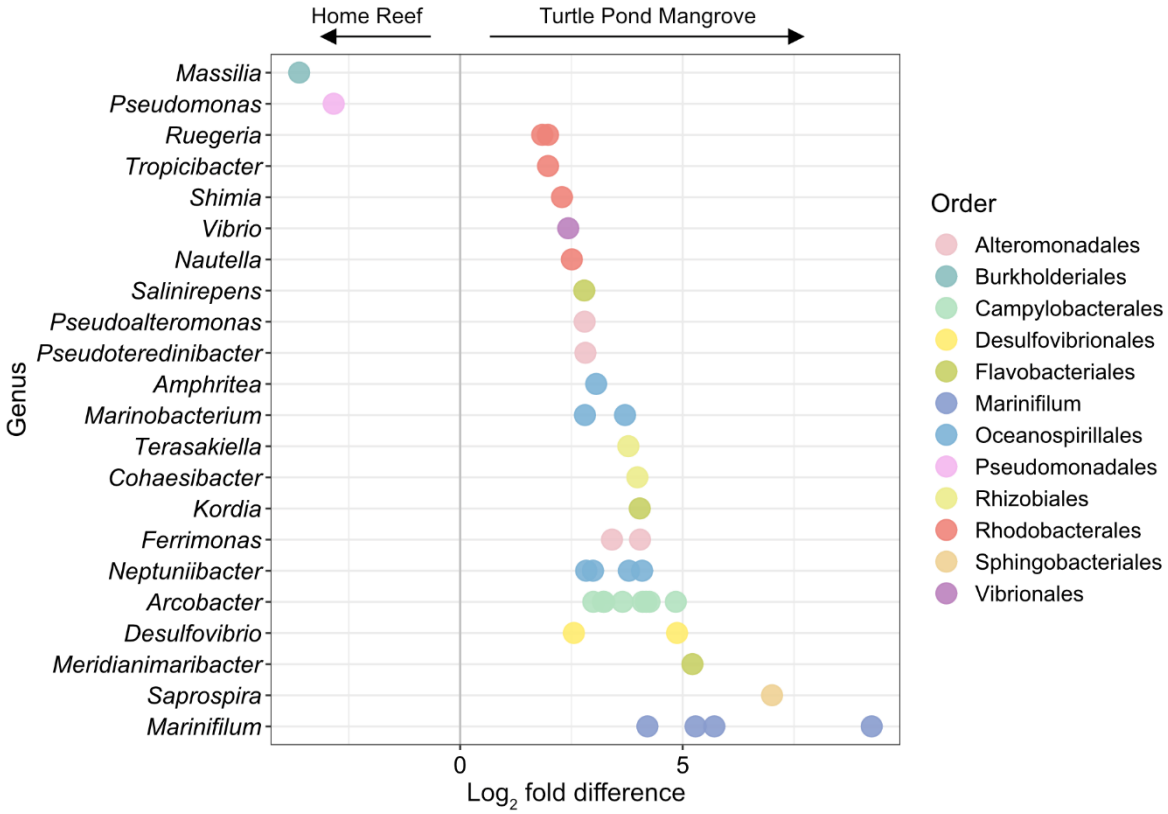


Figure 3.11. Differentially abundant bacterial OTUs associated with *Porites lutea* from Turtle Pond mangrove vs. Home Reef sampled (destination) sites. The $\log_2$ fold difference in geometric mean abundance is shown for significantly differently abundant OTUs (taxa more abundant in mangrove habitat on the right; taxa less abundant in mangrove habitat, and therefore more abundant in reef habitat on the left), using Wald test with Benjamini-Hochberg multiple-inference correction. Colours show which taxonomic Order OTUs belong to. Unclassified samples were removed. Analysis was performed using DESeq2.

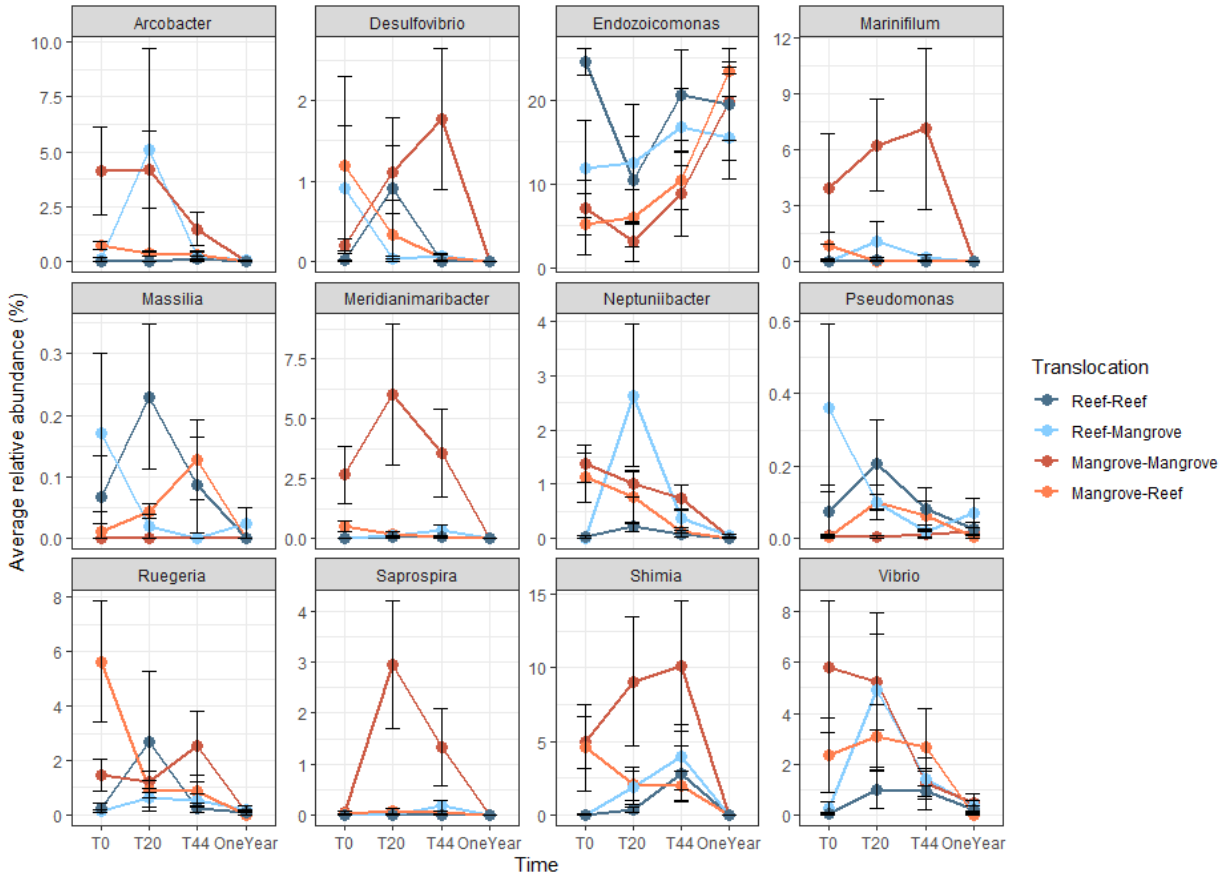


Figure 3.12. Relative abundance (%) of coral-associated bacterial genera most influenced by site (as determined by DESeq2), plotted over time. Values are mean relative abundance ± SE (*n* = 5). Colours represent the translocation treatment corals underwent.

Notably, over 30% of the bacterial community composition of mangrove to mangrove corals was comprised of only seven site-influenced genera (*Arcobacter:* 2-4%, *Desulfovibrio:* 1-2%, *Marinifilum:* 3-7%, *Meridianimaribacter:* 3-6%, *Saprospira:* 1-3%, *Shimia:* 5-10%, *Vibrio:* 2-6%) within two days of back-transplantation (Fig. 3.12). While the site-driven bacteria had decreased one year after translocation, the known coral symbiont *Endozoicomonas* increased in relative abundance (Fig. 3.12).

3.3.6. Coral – Symbiodiniaceae associations

Characterisation of the coral-associated Symbiodiniaceae revealed that colonies of *Porites lutea* from the mangrove generally hosted Symbiodiniaceae with different type profiles than those from the reef habitat; only type profile C15-C116ab-C15gu-C15gt-C116aa was

represented in both reef and mangrove corals before translocation (Fig. 3.13). By tracking the same coral colonies over time (Fig. 3.13), it was apparent that it generally took longer than two days (44 hours) to change algal symbionts, but that some colonies had switched symbionts after one year. Corals from the reef back-transplanted within the reef generally maintained a stable relationship with their algal symbionts; only one out of five colonies switched dominant symbiont type after one year (Fig. 3.13 A). Whereas three out of five reef corals translocated to the mangrove had switched symbiont type within a day (20h), and two of the five had different symbionts than they had started with one year after translocation (Fig. 3.13 A). Within two days of back-transplantation, all mangrove corals had the same dominant symbiont type, but after one year, three of these had switched. One of the coral colonies back-transplanted within the mangrove switched from hosting *Symbiodinium microadriaticum* (clade A1) to the more *Porites*-dominant *Cladocopium* sp. (clade C15) over the course of a year. All mangrove corals translocated to the reef also retained their dominant symbiont type within two days of translocation, and only one of the four relocated corals had switched after one year (Fig. 3.13 B).

In terms of ITS2 sequence diversity, SymPortal analysis yielded 186 sequence variants from 93 samples. There were 168 ITS2 sequence variants found associated with *P. lutea*, and 35 sequence variants associated with seawater. Only 16 ITS2 sequence variants were found in both coral and seawater samples. Multivariate analysis of variance revealed significant differences in ITS2 sequence diversity (richness, evenness, and Shannon-Wiener diversity) based on source habitat ($F_{(1,62)} = 4.76$, $P < 0.01$, Pillai's trace = 0.19) and destination habitat ($F_{(1,62)} = 5.28$, $P < 0.01$, Pillai's trace = 0.20). Reef origin corals hosted a higher diversity of ITS2 sequence variants (Fig. 3.14; richness ANOVA: $F_{(1,62)} = 11.57$, $P = 0.001$), and one year following translocation, the mangrove corals translocated to the reef had an increased relative abundance of rare ITS2 sequence variants compared with those which remained in the mangrove ($P < 0.05$) (Fig. 3.14).

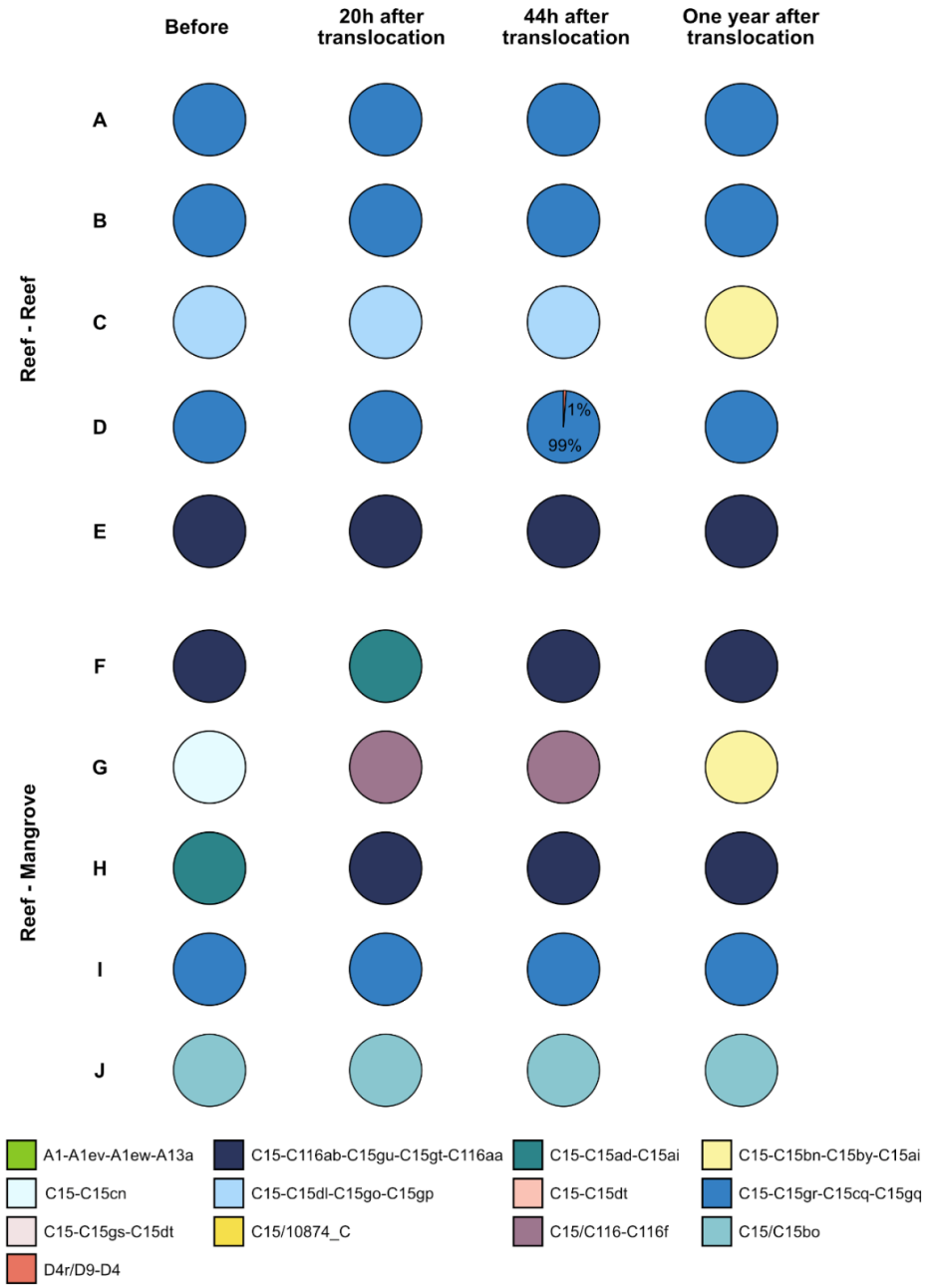


Figure 3.13. A) Symbiodiniaceae ITS2 type profiles of *Porites lutea*. Coral colonies are represented by letters (A-E for reef-reef transplants; F-J for reef-mangrove transplants). Each colony was sampled four times: before translocation, 20h after translocation, 44h after translocation and one year after translocation. The majority of samples comprised 100% one ITS2 type profile, but where more than one type profile was detected in a coral sample, the pie chart is annotated with the percentage composition of constituent type profiles. ITS2 type profile names are informative: capitalised letters denote the algal clade or genus of that putative taxon and hyphens separate the component defining intragenomic ITS2 sequence variants (DIVs) making up that profile, in decreasing order of abundance e.g. profile C15-C15dt refers to a genotype of clade C (*Cladocopium* genus), where the C15 sequence variant is most abundant, and C15dt sequence is next abundant. Symbiodiniaceae taxa characterised by co-majority abundances of component DIVs are denoted by a forward slash, e.g. C15/C15bo. Type profiles which contain sequence variants not already named in the literature have been assigned a numeric ID from the SymPortal database, e.g. C15/10874_C (Hume *et al.*, 2019).

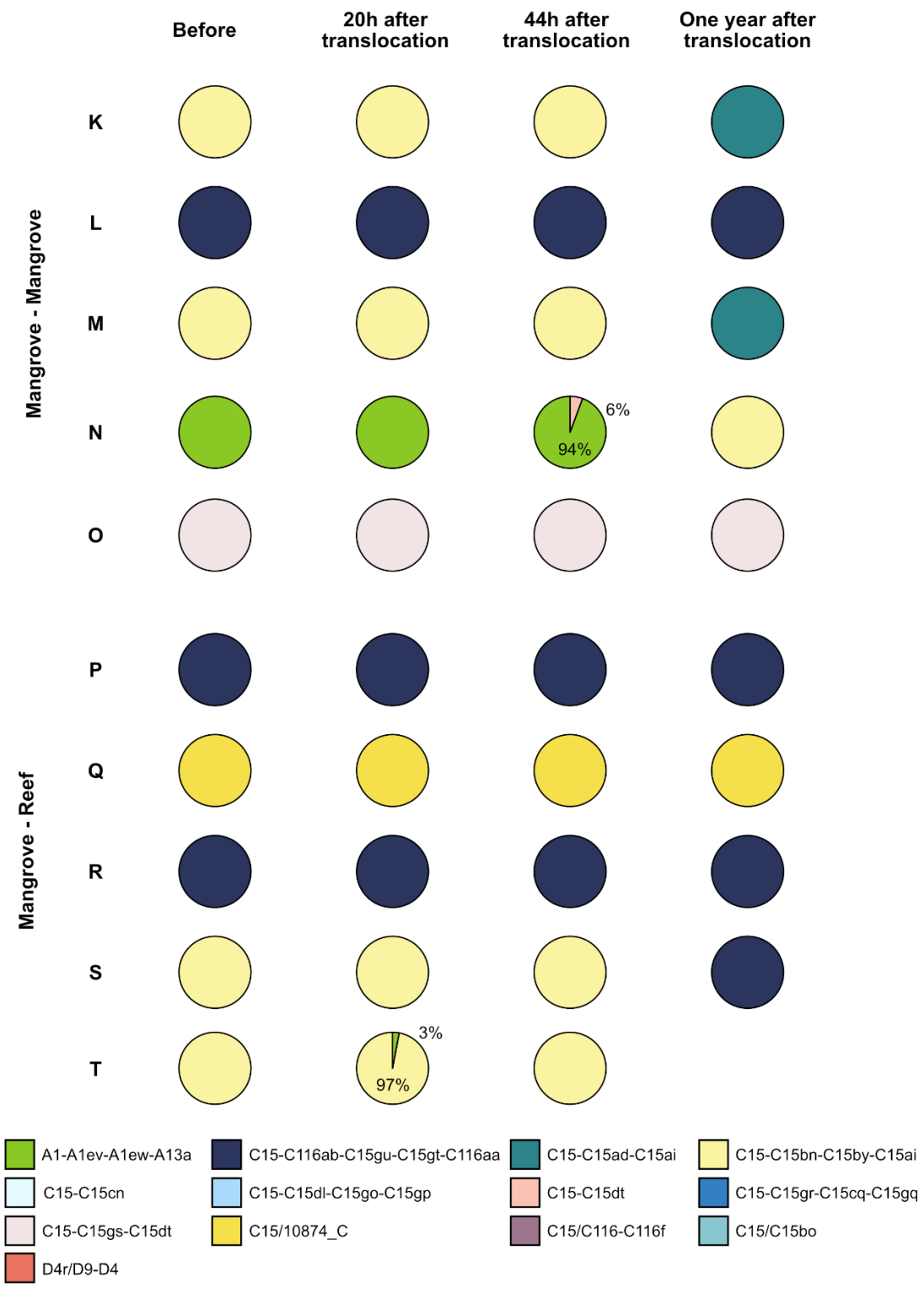


Figure 3.13. B) Symbiodiniaceae ITS2 type profiles of *Porites lutea*. Coral colonies are represented by letters (K-O for mangrove-mangrove transplants; P-T for mangrove-reef transplants). Each colony was sampled four times: before translocation, 20h after translocation, 44h after translocation and one year after translocation. The majority of samples comprised 100% one ITS2 type profile, but where more than one type profile was detected in a coral sample, the pie chart is annotated with the percentage composition of constituent type profiles.

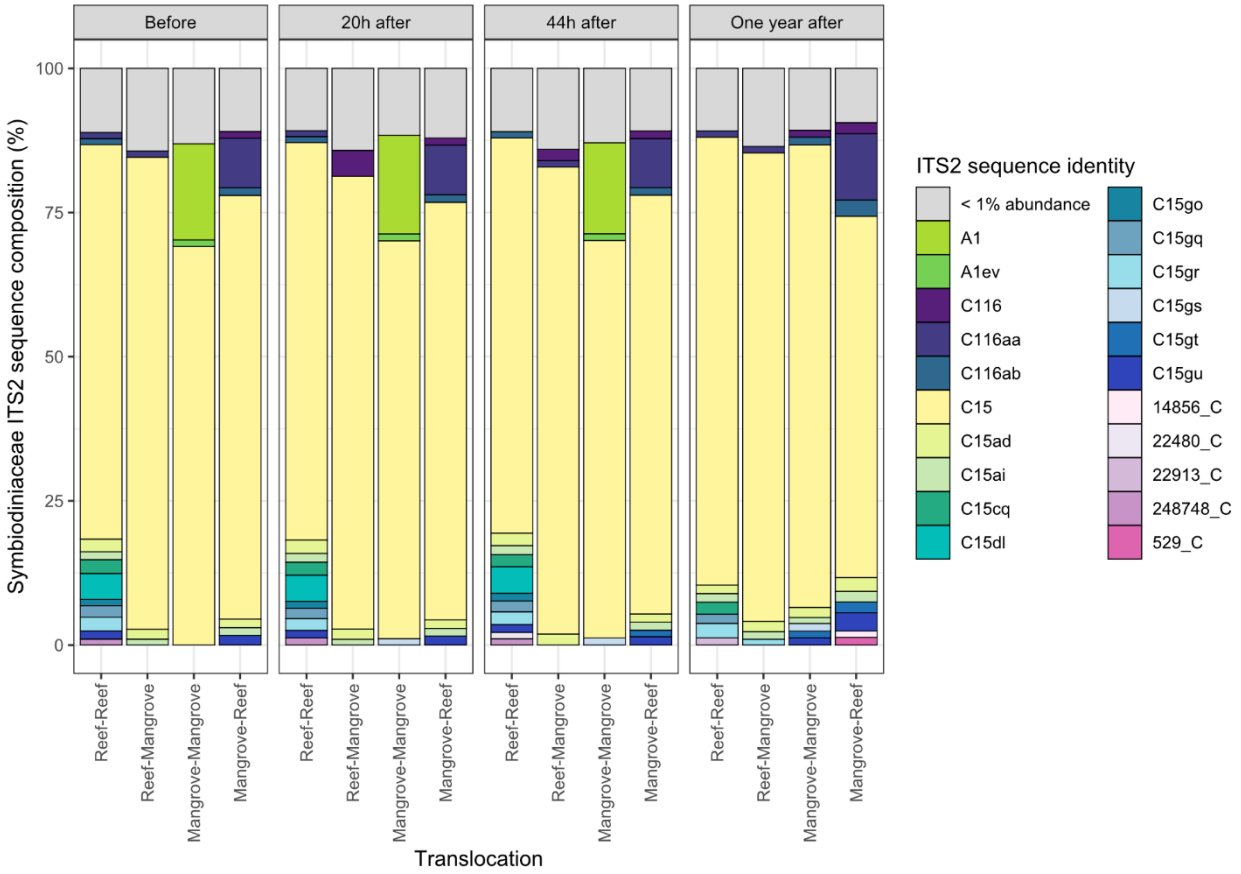


Figure 3.14. Average relative abundance (%) of Symbiodiniaceae ITS2 sequences of *Porites lutea* reciprocally translocated between mangrove and reef habitat. Panels show time points in the reciprocal translocation experiment (Before translocation, 20 hours post-translocation, 44 hours post-translocation, One year post-translocation). Colours represent the most abundant ITS2 sequence variants (> 1% mean abundance). Remaining sequences are grouped as '< 1% abundance'. Sequences commonly found in the literature or assigned a name through the SymPortal framework have their assigned names (e.g. C116, C15, or C15ad). Unclassified sequences are assigned a unique ID from the SymPortal database with the corresponding Symbiodiniaceae clade (e.g. 14856_C refers to a sequence with the unique ID 14856 from clade C, or *Cladocopium* genus.

3.4. Discussion

3.4.1. Marginal coral habitat resilience to marine heatwaves

Coral cover is not expected to be high in marginal coral habitats (Perry & Larcombe, 2003). However, the coral which was present in Turtle Pond mangrove (12% benthic cover in 2017) survived the Godzilla El Niño of 2016, while corals on the reef perished (Gardner *et al.*, 2019). Benthic transect data from 2017 and 2018 supported that the reef, with higher dead

coral cover, was more susceptible to the deleterious effects of marine heatwaves (Fig. 3.4). The difference in resilience between the two habitats was emphasised by the persistence of branching coral taxa such as *Acropora* spp. (generally considered susceptible to heat stress; Marshall & Baird, 2000; Loya *et al.*, 2001) in the mangrove, versus the post-El Niño dominance of stress-tolerant taxa, such as *Porites lutea* and *Pavona clavus,* on the reef. Turbid shallow reef communities have previously proven to be surprisingly resilient to major thermal anomalies, such was the case for a highly disturbed reef in Singapore (Guest *et al.*, 2016).

3.4.2. Coral-associated microbial abundance is highly variable

The microbial abundance associated with *Porites lutea* was too variable to draw conclusions on the impact of habitat or translocation (Fig. 3.6). Coral-associated bacterial abundances, determined by qPCR, spanned five orders of magnitude (10^7 to 10^{12} 16S rRNA gene copies g^{-1} coral tissue). Previous studies cite bacterial abundance estimates around 10^8 bacteria cm^{-2} coral tissue when enumerated by SYBR gold staining (Koren & Rosenberg, 2006), or 10^6 cells cm^{-2} enumerated by DAPI staining and confocal microscopy (Garren & Azam, 2012). Abundance estimations of coral-associated microbial aggregations within tissues have been around 10^4 cells cm^{-2} by fluorescence *in situ* hybridisation (FISH; Wada *et al.*, 2019). Bacterial densities in coral mucus have been measured between 10^5 cells ml^{-1} (Garren & Azam, 2010) to 10^8 cells ml^{-1} by DAPI staining (Garren & Azam, 2012). Quantitative real-time PCR (qPCR) has been suggested as one of the best methods to measure coral-associated microbial abundances as taxa-specific genetic markers can be developed (Cunning & Baker, 2014). As with all methods, there are some pitfalls, including the effect of DNA extraction efficiency, inaccurate standardisation to mass of sample extracted from, variability between technical replicates due to the logarithmic calculations from quantitation cycles (Cq), and copy number in the genome for target loci, as well as the

need to design primers which mis-match non-target sequences (Mieog *et al.*, 2009a; Cunning & Baker, 2013). Primer specificity was an issue for quantifying coral-associated fungi in this study. The gold standard for accurate estimation of microbial loading would be to standardise against a single copy coral host gene (such as the low copy-number actin gene loci; Mieog *et al.*, 2009) to estimate abundance of microorganisms per coral host cell, though this requires meticulous development of unique primers for each coral host species (*Acropora millepora*: Mieog *et al.*, 2009; *Pocillopora damicornis*: Cunning & Baker, 2013; *Montastraea cavernosa*: Silverstein *et al.*, 2015; *Orbicella faveolata* and *Siderastraea siderea*: Cunning, 2013). As such, the microbial abundance estimates given here can be compared between groups in this experiment, but absolute numbers (particularly for Symbiodiniaceae which were based on the multi-copy ITS2 loci) are not accurate estimates of cell density.

Environmental samples often have very variable microbial densities due to environmental heterogeneity over space and time. The variability in microbial abundance of corals measured in this study could have been the result of both within and between colony variability. Of the few studies which have reported spatial distribution of microorganisms across a coral surface, *Porites furcata* and *Orbicella annularis* exhibited spatial heterogeneity (Rohwer *et al.*, 2002; Daniels *et al.*, 2011), while *Acropora palmata* did not (Kemp *et al.*, 2015). Microhabitats within coral colonies have been better studied in the context of skeletal architecture (Yost *et al.*, 2013) and light conditions for the algal symbionts (Wangpraseurt *et al.*, 2014). Since corals are known to shed bacteria with their mucus (Garren & Azam, 2012; Glasl *et al.*, 2016), the variability measured between colonies here could be due to differences in sampling time since last mucus shed, and thus differences in the stage of ecological succession of the coral surface microbial assemblage. This may be especially pertinent for *Porites* species as they form ‘mucus tunics’ which age before sloughing away under wave action (Coffroth, 1990; Brown & Bythell, 2005).

Coral-associated bacterial abundance did not vary according to site, which corroborates findings from previous studies; *Porites lobata* exposed to chronic nutrient enrichment by a wastewater treatment plant (Garren & Azam, 2010), and *Porites cylindrica* exposed to fish farm effluent (Garren *et al.*, 2009), exhibited no difference in bacterial loading compared with oligotrophic reference reefs. There may be a carrying capacity to microbial loading of coral tissues and surfaces regulated by the host, with nutrients in the coral mucus limiting microbial population growth rather than external environmental nutrient concentrations. It is important to remember that the coral microbiome, and all microbial assemblages, are highly dynamic; while certain microbial taxa decrease in abundance, others will take their place. Therefore, microbial abundance alone is not indicative of differences in richness or diversity of microbial communities between habitats, as absolute abundance does not reflect any compositional differences.

3.4.3. Distinct coral and water bacterial communities

It is now well-established that corals host distinct microbiomes which are different to the overlying seawater (Rohwer *et al.*, 2001; Frias-Lopez *et al.*, 2002; Bourne & Munn, 2005), and in this study it was also observed that the bacterial community composition of *Porites lutea* was distinctly different to that of the surrounding seawater in both habitats (Fig. 3.7). The coral surface mucus layer (SML) provides a very different habitat for microorganisms compared with the surrounding seawater, in terms of viscosity and nutrient provision (Brown & Bythell, 2005; Bythell & Wild, 2011). Corals are known to have an innate immune system which can keep foreign microorganisms out (Palmer, 2018). Several mechanisms have been proposed by which the coral holds control over the microbial community it plays host to (Krediet *et al.*, 2013). Purported mechanisms include 1) a host ability to detect microbe-associated molecular patterns and subsequent defences to exclude undesirable microorganisms (van de Water *et al.*, 2018a), like the establishment of the *Euprymna* squid-

Vibrio symbiotic relationship (Nyholm & McFall-Ngai, 2004), 2) excretion of antimicrobial compounds (Ritchie, 2006), 3) release of chemical cues and nutrients to attract potentially beneficial microbes by chemotaxis (Tout *et al.*, 2015a), and 4) maintenance of a community of beneficial microorganisms so that they, in turn, engineer the microbiome and prevent invasion by environmental opportunists (Nissimov *et al.*, 2009; Kvennefors *et al.*, 2012; Raina *et al.*, 2016).

The difference in seawater bacterial communities between mangrove and reef habitat was largely driven by the higher relative abundance of Rhodobacteraceae in mangrove water. The Rhodobacteraceae are known to be common in marine environments and include bacteria with diverse metabolic capabilities including chemo- and photoheterotrophs, as well as several known aquatic symbionts (Pujalte *et al.*, 2014). While there is potential for horizontal transmission of microorganisms from the surrounding seawater, it is not simply the case that whatever is there will penetrate the coral surface.

3.4.4. Coral-associated bacterial communities exhibit environmental plasticity

This study of microbial communities associated with corals from a marginal mangrove habitat revealed the influence of habitat on the coral-associated bacterial community of *Porites lutea*. Before translocation, mangrove and reef corals hosted distinct bacterial communities. While there was no difference in the diversity of the coral-associated bacterial communities, nMDS ordination showed a clear distinction in the bacterial community composition between corals of different habitats (Fig. 3.9).

3.4.4.1. Bacterial diversity was no different for mangrove vs. reef corals

Greater bacterial diversity is often linked to corals exhibiting disease symptoms (Pantos & Bythell, 2006), corals with microbiomes disrupted by opportunistic microorganisms (Garren *et al.*, 2009), or corals living in stressful, degraded, or human-influenced environments (Ziegler *et al.*, 2016; McDevitt-Irwin *et al.*, 2017; Claar *et al.*, 2020). However, there was no difference in bacterial alpha diversity between corals from reef and mangrove habitats before translocation. Coral colonies from the reef varied more between one another in terms of OTU richness and diversity than corals from the mangrove. One plausible explanation for this could be that the fore-reef was a more environmentally patchy habitat with more heterogeneous substrate cover, which caused some corals to host highly biodiverse bacterial communities, while others had low bacterial diversity. This contrasts to the perpetually extreme conditions of the mangrove which instigated consistently more even, and perhaps more disturbed, bacterial communities. Generally, coral-associated bacterial OTU richness and evenness decreased with time following transplantation (Fig. 3.8). This was concomitant with an increase in relative abundance of the known coral endosymbiont, *Endozoicomonas* in the family Hahellaceae (Fig. 3.10 & Fig. 3.12). Since high relative abundances of *Endozoicomonas* are usually associated with healthy non-stressed corals (Bayer *et al.*, 2013a; Pootakham *et al.*, 2019), the increase in relative abundance of *Endozoicomonas* in *Porites lutea* sampled one year after translocation could signify a reduction in stress since transplantation and acclimatisation to life in a new habitat, though bacterial diversity did decrease to levels lower than pre-translocation. The decreased diversity one year after transplantation cannot be explained by sequencing depth, as this was accounted for by rarefaction. A methodological consideration, and potential limitation, of transplantation studies is that the act of transplantation itself introduces stress and can trigger a shift in the coral microbiome (Casey *et al.*, 2015). It is also possible that the bacterial communities were affected by a common change in the environment which was not

captured, and/or corals could have inadvertently been sampled soon after mucus shedding (Glasl *et al.*, 2016).

3.4.4.2. Bacterial community composition of *Porites lutea* was habitat-driven

As has been reported in several studies of healthy corals, colonies of *Porites lutea* from both habitats were dominated throughout the study period by the bacterial family Hahellaceae which includes the known endosymbiont *Endozoicomonas* (Fig. 3.10 & Fig. S3.3). *Endozoicomonas* are believed to be an important taxon of the coral microbiome as they are consistently prevalent across coral species worldwide (Huggett & Apprill, 2019), and have been recorded in high abundance in corals from healthy reefs (Bayer *et al.*, 2013b; Bourne *et al.*, 2016). Following interrogation of the *Endozoicomonas* genome, they are purported to play important roles in carbohydrate cycling and provision of protein to the host, and may have co-diversified with their coral host species (Neave *et al.*, 2017a, 2017b). *Endozoicomonas* genotypes have been found to exist with certain coral hosts (Neave *et al.*, 2017b), and habitats: with different genotypes found to associate with *Acropora* located in mangrove and reef sites (Camp *et al.*, 2020). Corals in this study were generally dominated by two Hahellaceae taxa. The first, OTU 1 (unclassified Hahellaceae) identically matched to a sequence in the NCBI database from a healthy colony of *Porites lutea* in Mayotte, Western Indian Ocean (accession: KF179705), and its nearest cultured relative was a symbiont of the *Loripes lacteus* clam with 96.56 % similarity (GQ853556). The second, OTU 3 (*Endozoicomonas* sp.) matched a sequence from the coral *Pavona duerdeni* in Koh Tao, Thailand (KC527076), while the closest cultured sequence was *Endozoicomonas gorgoniicola* (96.58% identity; NR_109685) isolated from the octocoral *Plexaura* sp. in the Bahamas. One coral colony from the mangrove (colony O) consistently associated with a *Kistimonas* (OTU 98; family Hahellaceae) in greater abundance than an *Endozoicomonas,* and also hosted a different algal endosymbiont (type profile: C15-C15gs-C15dt). The same

Kistimonas sequence was previously found in *Porites lutea* from South Africa exhibiting *Porites* White Patch Syndrome (KF180031; Séré *et al.*, 2013). Other *Kistimonas* have previously been isolated from marine invertebrates such as starfish (Choi *et al.*, 2010), clams (Lee *et al.*, 2012), and ragworms (Christopher Ellis *et al.*, 2019).

Aside from Hahellaceae, the bacterial community composition hosted by *Porites lutea* was markedly different for corals living in different habitats (Fig. 3.9 & Fig. 3.10). Corals from the mangrove hosted higher abundances of the potentially opportunistic bacterial families Campylobacteraceae, Vibrionaceae, and Rhodobacteraceae (Tout *et al.*, 2015b; Gignoux-Wolfsohn *et al.*, 2017), as well as other known coral-associated bacterial families, including Alteromonadaceae, Colwelliaceae, Marinifilaceae, and Oceanospirillaceae. Despite commonly being found in coral microbiomes, Campylobacteraceae have previously been implicated as one of the candidate causative agents of White Band Disease (Gignoux-Wolfsohn & Vollmer, 2015), and Black Band Disease (Frias-Lopez *et al.*, 2002), and have also been found to increase in abundance following wounding and exposure to fish faeces (Ezzat *et al.*, 2019). There were ten phylotypes of the Campylobacteraceae genus, *Arcobacter*, which were significantly differentially more abundant in corals sampled from the mangrove (Fig. 3.11). *Arcobacter* has previously been detected in *Porites cylindrica* transplants exposed to fish farm effluent (Garren *et al.*, 2009), and is commonly cited as being pathogenic (Frias-Lopez *et al.*, 2002). However, it has also been cited as a core member of the *Pocillopora damicornis* holobiont*,* due to its prevalence in a majority of samples across mitochondrial lineages of the host, and in different geographic regions displaying different thermal regimes (Brener-Raffalli *et al.*, 2018). Rapid increases in *Arcobacter* abundance resulting from thermal stress (Shiu *et al.*, 2017) reinforce its reputation as an opportunistic bacteria. Members of Vibrionaceae, whilst also common component taxa of coral microbiomes (Huggett & Apprill, 2019), are also infamously known to play roles in bacterial bleaching and disease (Ben-Haim *et al.*, 2003; Arotsker *et al.*,

2009). As opportunistic bacteria, these taxa are not harmful in low numbers, but have the potential to become pathogenic under certain conditions, such as *Vibrio coralliilyticus* under elevated temperatures (Ben-Haim *et al.*, 2003; Kimes *et al.*, 2012).

The bacterial family Rhodobacteraceae is also often referred to as opportunistic (Mouchka *et al.*, 2010; Ziegler *et al.*, 2016; McDevitt-Irwin *et al.*, 2017), and has been linked with aged mucus (Glasl *et al.*, 2016), and thermally stressed *Porites lutea* (Pootakham *et al.*, 2019). The relatively higher abundance of Rhodobacteraceae in mangrove seawater and corals may reflect the more stressful and fluctuating conditions of the mangrove compared with the reef, or as previously mentioned, Rhodobacteraceae may be able to take advantage of energy sources not usually present in oligotrophic reef settings, due to their diverse metabolic capabilities (Pujalte *et al.*, 2014). Key phylotypes which were found in higher abundance in mangrove-sampled corals included a *Shimia* (OTU 12), a *Nautella* (OTU 105), and two *Ruegeria* (OTU 793 and OTU 73817). Exact matches for the *Shimia* phylotype OTU 12 had previously been found associated with the anemone *Exaiptasia pallida* (KY347063), hard coral *Acropora hemprichii* (MK736223), and *Litopenaeus vannamei* shrimp (MK589157), and the closest cultured relatives were *Shimia isoporae* (MH283808; Chen *et al.*, 2011) and *Shimia marina* (MG707630; Choi & Cho, 2006). While both *Shimia* and *Ruegeria* have been commonly associated with stressed and diseased corals, and referred to as opportunistic pathogens (Godwin *et al.*, 2012; Meyer *et al.*, 2019; Pootakham *et al.*, 2019), *Ruegeria* may actually provide a mutualistic role when its coral host is faced with heat stress. Three strains of *Ruegeria* from the coral *Galaxea fascicularis* were found to inhibit the growth of the temperature-dependent pathogen *Vibrio coralliilyticus* (Miura *et al.*, 2019), and *Ruegeria* was found to be a bioindicator of corals inoculated with the pathogen (Rosado *et al.*, 2019). Since *Ruegeria* spp. have shown such promise as defensive symbionts, novel primer sets have been developed for their accurate detection (Kitamura *et al.*, 2020).

Before translocation, Alteromonadaceae were found in naturally greater relative abundance in association with corals from the mangrove than with corals from the reef. In a study of depth-generalist corals, one member of the family Alteromonadaceae was found to persistently associate with > 98% of coral colonies, across a depth range of 10 to 80 m, suggesting it was a core member of the coral microbiome (Hernandez-Agreda *et al.*, 2018). Other members of the Alteromonadaceae have also been found in the very early life stages of several coral species suggesting they may have important functional roles and provide benefits to vulnerable settling corals (Sharp *et al.*, 2012; Ceh *et al.*, 2013; Damjanovic *et al.*, 2020), which could also be advantageous for survival in a mangrove environment.

Other indicator phylotypes associated with corals living in mangrove habitat included a *Meridianimaribacter* (family Flavobacteriaceae; OTU 30), and sulfate-reducing *Desulfovibrio* (Widdel & Bak, 1992; family Desulfovibrionaceae; OTU 275 and OTU 631), both of which can metabolise recalcitrant substrates and access nutrients otherwise unattainable by the coral host. The first genomic analysis of a halotolerant *Meridianimaribacter* isolated from mangrove soil revealed the presence of genes encoding lignocellulose-degrading enzymes such as cellulases, xylanases, and mannanases (Lam *et al.*, 2020). Such metabolic capabilities could be an asset for converting readily available woody plant matter in a mangrove to a viable carbon source for the coral host. A reliance on bacterial nutrient acquisition by corals living in sub-optimal conditions was previously proposed as an explanation for the high diversity of bacteria found to associate with corals on mesophotic reefs (Hernandez-Agreda *et al.*, 2016a).

While there are not yet enough studies of coral-associated bacterial communities from mangrove habitats to draw conclusions on the existence of a core mangrove coral microbiome, this study suggests that the coral microbiome is influenced by, and can be changed by, the conditions presented by a mangrove habitat, with differences driven by key bacterial taxa. There were over ten times more bacterial phylotypes significantly indicative of

mangrove habitat (DESeq2 differential abundance analysis) than there were of reef habitat, which echoes findings from Bouraké mangrove lagoon, New Caledonia (Camp *et al.*, 2020). Environmental plasticity in coral microbiome composition has similarly been documented for corals thriving in the warm waters of the northern Red Sea (Osman *et al.*, 2020).

3.4.4.3. Bacterial communities of *Porites lutea* were spatially and temporally flexible

The concept of coral-associated bacterial flexibility in terms of community composition is relatively new, and implies that different coral species are capable of differing levels of microbiome flexibility (Pogoreutz *et al.*, 2018; Ziegler *et al.*, 2019; Voolstra & Ziegler, 2020). This experiment highlighted that the *Porites lutea* microbiome is flexible and capable of fast microbial turnover. Within 20 hours of translocation to a new habitat, the once distinct communities had become highly similar (Fig. 3.9). The rapid habitat-driven change in coral-associated bacterial community observed in this study is supported by findings from the corals *Pachyseris speciosa*, *Mycedium elephantotus*, and *Acropora aculeus*, where a large proportion of the coral microbiome (estimated at > 96% of bacterial phylotypes) is environmentally responsive, and not constrained by the coral host (Hernandez-Agreda *et al.*, 2018). Previous translocation experiments have shown that corals are capable of this microbiome flexibility, where transplants match the microbial community composition of conspecifics native to the sampled environment; though this was studied following much greater timescales (17 months: Ziegler *et al.*, 2017; 21 months: Ziegler *et al.*, 2019).

It is worth remembering that DNA sampling only ever represents a snapshot in time for a microbial community. In this study, indicator taxa of corals sampled from mangrove habitat increased suddenly, before decreasing rapidly (Fig. 3.12). This illustrates the dynamic and sporadic nature of the environmentally-responsive coral microbiome. Coral-associated bacterial sampling will have been subject to natural phenomena such as tidal cycles and

mucus shedding, as well as bacterial interactions following colonisation, such as competition and antagonism. It is likely that the corals inhabiting the mangrove habitat must also contend with a constant regular influx of opportunistic bacteria with the tide stirring up fine sediments. Coral microbiomes are already known to be tidally influenced (Sweet *et al.*, 2017b).

A common attribute shared by many of the mangrove habitat indicator taxa was their link to opportunism and disturbed coral microbiomes. Whilst microbiome flexibility could lead to opportunities for rapid acclimatisation/adaptation to fast-changing environmental conditions (Reshef *et al.*, 2006), it could also signal a disturbed or stressed microbiome with opportunistic bacteria taking advantage where they can. A major question therefore is whether corals in the mangrove are thriving or surviving? While *Porites lutea* was able to change its associated microbiota based on the local environment, presumably at least partially through uptake from the new environment (horizontal transmission), it remains unclear what advantages (or disadvantages) this might have conferred. *Acropora hyacinthus* colonies translocated to a more thermally variable environment changed microbiome structure to match native corals living in the environment, and simultaneously developed superior thermal tolerance, though the authors of the study could not be sure whether bacteria were responsible (Ziegler *et al.*, 2017). Similar to a study of human-impacted reefs in the Red Sea (Ziegler *et al.*, 2016), the findings presented here can be interpreted in two ways. Either the recorded shifts in coral microbial community provide support to the Coral Probiotic Hypothesis, whereby microorganisms from the mangrove assist in rapid adaptation to the environment (Reshef *et al.*, 2006), or corals living in the mangrove have disturbed microbiomes existing at a tipping point towards a diseased state. A further hypothesis could be that since the microbiomes of the mangrove corals are heavily site-influenced, and potentially regularly exposed to opportunistic pathogens, it provides the mangrove corals with an opportunity to regularly practice immune responses. Corals and other Cnidaria have exhibited evidence of immune memory or immunological priming (Brown & Rodriguez-

Lanetty, 2015; Palmer, 2018), which could render mangrove corals more prepared for large scale stressor events, such as thermal anomalies, which might disrupt the coral microbiome. Alternatively, the mangrove corals might host microorganisms capable of antimicrobial action or antagonistic interactions against invading pathogens.

3.4.5. Algal symbiont specificity and stability

As with the coral-associated bacterial assemblages, the algal symbiont communities also varied with habitat. Based on ITS2 type profile, only one putative taxon (C15-C116ab-C15gu-C15gt-C116aa) was shared between *Porites lutea* of mangrove and reef habitat, before translocation. Such distinctions in symbiont genotypes between *Porites lutea* from mangrove and reef habitats are in agreement with reports from the Great Barrier Reef (Camp *et al.*, 2019) and New Caledonia (Camp *et al.*, 2020). However, in contrast with the flexible and transient bacterial community, the Symbiodiniaceae showed greater host-fidelity, and remained more stable over time (Fig. 3.13). Of the twenty *P. lutea* colonies in this experiment, the only three colonies which changed dominant symbiont taxa within two days were those which had been translocated from the reef to the mangrove, perhaps suggesting that the mangrove exerts greater selective pressure than the reef. Camp *et al.* (2019) hypothesised that flexibility in the coral-Symbiodiniaceae relationship might allow coral holobionts to meet their metabolic demands when living in the different ‘resource landscape’ of mangroves compared with reef habitat.

Generally, *Porites lutea* maintained association with symbionts of the *Cladocopium* genus, particularly the C15 lineage or ‘sub-clade’. The association between Indo-Pacific *Porites* and *Cladocopium* is well known (LaJeunesse, 2005; Fitt *et al.*, 2009; Barshis *et al.*, 2010). *Porites lobata* inhabiting both thermally extreme back-reef and more stable fore-reef habitat in American Samoa were also found to associate with the symbiont *Cladocopium* C15 (Barshis *et al.*, 2010). Furthermore, sub-clade C15 was described as heat-resistant following

a short term thermal stress experiment with *Porites cylindrica* (Fitt *et al.*, 2009). The putative symbiont taxa found to associate with *Porites lutea* in this study (Fig. 3.13) could be similar to those found in Woody Isles mangroves, Great Barrier Reef (type profile C15-C15by-C15bn; Camp *et al.*, 2019) and Bouraké mangrove, New Caledonia (type profiles C15, C15-C15az, and C15-C15bn; Camp *et al.*, 2020), which begs the question of whether there are certain algal symbiont genotypes which are specifically adapted to extreme mangrove conditions. The stable associations between coral host colony and symbiont genotype over time align with the known mode of symbiont transmission for *Porites lutea*; *Porites* is known to be one of few spawning coral genera which passes its symbionts on via vertical transmission (from parent colony to eggs; Baird *et al.*, 2009), so coral-symbiont associations may have persisted since before settlement.

There was one coral colony, from the mangrove, which associated with a genotype of *Symbiodinium microadriaticum* (ITS2 type profile: A1-A1ev-A1ew-A13a), but which over the course of a year switched to the more typical *Porites-Cladocopium* association. *Porites lutea* in the Red Sea has previously been shown to form flexible associations with symbionts, which changed between summer and winter (Ziegler *et al.*, 2015). The conclusion of that study was that coral host species might either associate with one specific symbiont taxon with broad physiological tolerance (e.g. *S. microadriaticum* aka A1), or hosts will associate with multiple more specialised symbiont taxa over time, to suit the prevailing conditions (Ziegler *et al.*, 2015). Despite notoriously being linked to enhanced thermal tolerance (LaJeunesse *et al.*, 2014; Silverstein *et al.*, 2017), the symbiont *Durusdinium* (type profile: D4r/D9-D4) was only hosted in low abundance (1%) by one reef coral. This highlights the need to further investigate genotypes of other endosymbiont genera, which may be of greater importance in hyper-variable extreme marginal coral habitats.

Changes in algal symbionts generally took longer than two days (Fig. 3.13), and based on the putative taxa generated by SymPortal (ITS2 type profiles), it seemed symbiont switching

(from one dominant symbiont type to another) was the most prevalent mode of change. However, when assessing ITS2 sequence variants alone, it looks as though corals remained dominated by one C15 sequence but shuffled relative abundances of other rare sequence variants over time (Fig. 3.14). This highlights the importance of methodology for establishing taxonomic units/ biological entities when sequencing multi-copy genetic regions such as ITS2. Clearly different methods can render different interpretation of results. As such, it would be interesting to revisit and update previous studies which typed to symbiont clade level regarding the phenomena of symbiont switching versus shuffling (Goulet, 2006; Cunning *et al.*, 2015).

Corals from the mangrove generally hosted a lower diversity of ITS2 sequence variants (Fig. 3.14). While this may seem counterintuitive in the face of multiple fluctuating stressors in the mangroves, corals in a marginal non-reef environment in Hong Kong have also been found to host a reduced diversity of symbiont types, dominated by *Cladocopium* C1, when compared with neighbouring sites (Ng & Ang, 2016). The authors suggested it could be an adaptive strategy to cope with fluctuating stressful conditions. A modelling study of *Porites lutea*-Symbiodiniaceae associations across southeast Asia found that high variance in SST correctly predicted reduced endosymbiont diversity (Tan *et al.*, 2020). These findings suggest that environments with extreme fluctuating conditions exert a strong selective pressure on endosymbiont types, such that only those with wide-ranging tolerance limits can persist. It might also be the case that it becomes too costly for the coral to host multiple specialised symbiont types and be constantly switching, so the most viable strategy is to associate with one type which can cope with a breadth of abiotic conditions. Host-specificity of algal endosymbionts (in contrast to flexible coral-bacterial assemblages) across a latitudinal gradient in the northern Red Sea also hinted at high physiological plasticity by Symbiodiniaceae (Osman *et al.*, 2020). Further experiments are warranted to characterise the physiological capabilities of distinct Symbiodiniaceae genotypes.

3.4.6. Local adaptation

A broader question of this study, and often the focus of reciprocal translocation studies (Ågren & Schemske, 2012; Berggren *et al.*, 2016), was whether conspecific corals were locally adapted to their source habitats, and whether there would be any reduced survival or trade-offs for living in a new environment. This study provided little evidence of local adaptation. While none of the translocated corals died following a year in a new environment, half of the colonies translocated from the mangrove to the reef exhibited signs of bleaching on part of the colony (Table 3.3). Therefore, the *Porites lutea* holobiont may be locally adapted to the mangrove environment such that adaptations to extreme mangrove conditions come at a cost of poorer resilience in what is thought to be a more benign reef environment. *Porites lutea* is known to be an especially stress-tolerant coral which is often reported to have survived or recovered from anomalously high temperatures (Loya *et al.*, 2001; van Woesik *et al.*, 2011). Since this study was conducted one year after the mass-bleaching event of 2016, and *Porites lutea* is a slow-growing coral, all the colonies included in this study had survived anomalously high SSTs and therefore must have been inherently thermally tolerant. Without measures of coral health, it is difficult to visually assess whether a coral is thriving or surviving in its environment. However, by amplicon sequencing the coral-associated microbiota, it became apparent that there was a local coral-associated bacterial community. Future experiments involving coral transplantation into and out of marginal coral habitats could go further to study various fitness traits such as growth, calcification, and metabolic rates, in cross-transplants versus local transplants.

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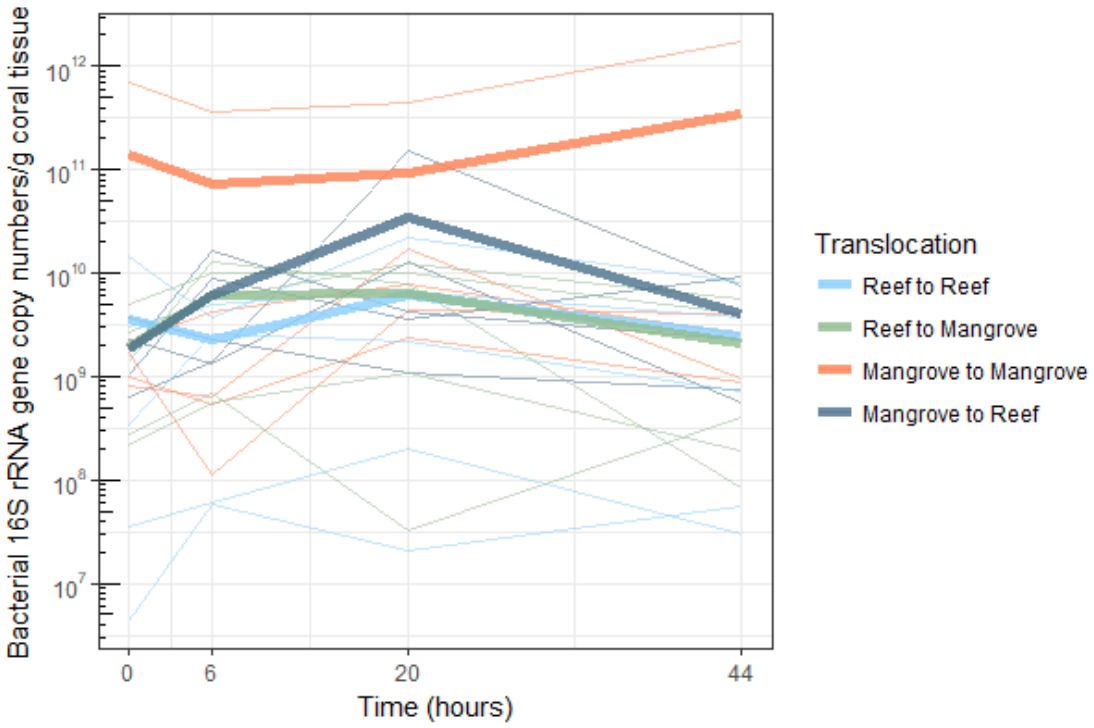
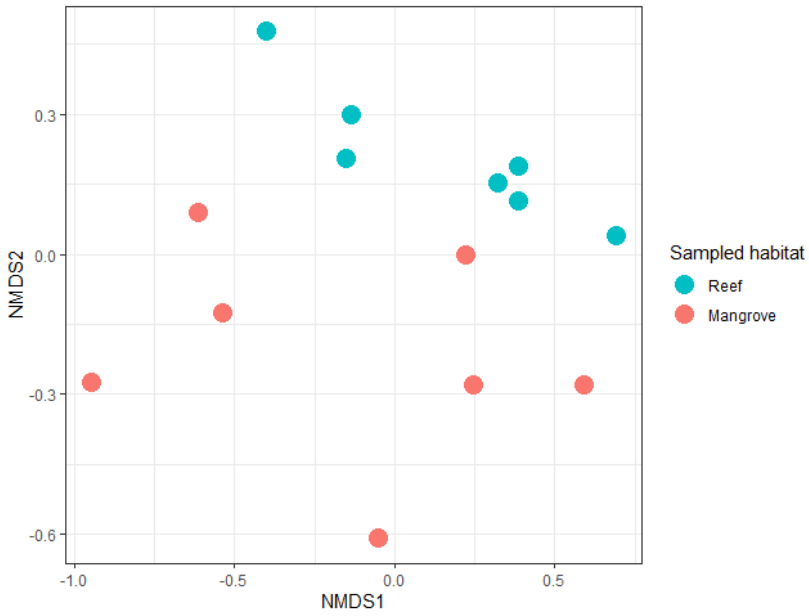
3.7. Supplementary material



Supplementary figure 3.1. Thin lines represent the bacterial loading of individual coral colonies over time. Thick lines are mean bacterial load, coloured by translocation. Colony L back-transplanted from mangrove to mangrove hosted much higher bacterial loads, which skewed the mean for this group.



Supplementary figure 3.2. Non-metric Multi-Dimensional Scaling ordination of seawater bacterial communities from reef habitat (blue) and mangrove habitat (orange).

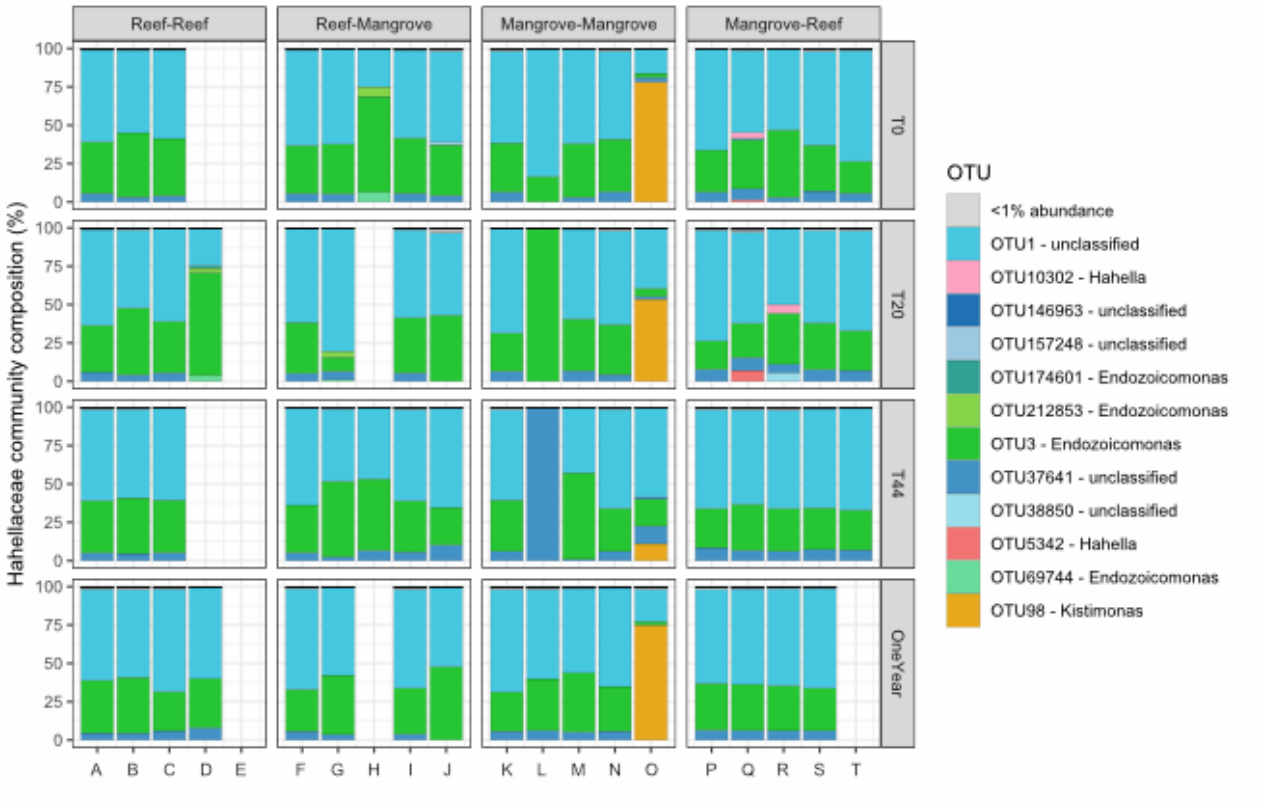
Supplementary table 3.1. Permutational analysis of variance (PERMANOVA) of bacterial communities hosted by *Porites lutea*. Model specified as source (levels: reef vs. mangrove) by transplantation (levels: back-transplanted vs. cross-transplanted), over time.

Three-way PERMANOVA				
Factor	**Model-F**	**R^2**	**Df**	**P_{MC}**
Source site	2.959	0.037	1	< 0.05
Transplantation	1.395	0.017	1	0.192
Time	3.436	0.129	3	< 0.001
Source × Transplantation	2.484	0.031	1	< 0.05
Source site × Time	1.393	0.052	3	0.135
Transplantation × Time	0.502	0.013	2	0.947
Source × Transplantation × Time	0.787	0.020	2	0.616

Pairwise comparisons of Source site × Transplantation				
	Back-transplanted reef coral	**Cross-transplanted reef coral**	**Back-transplanted mangrove coral**	**Cross-transplanted mangrove coral**
Back-transplanted reef coral	-	0.865	3.802	0.983
Cross-transplanted reef coral	1.000	-	1.960	0.634
Back-transplanted mangrove coral	< 0.05	0.528	-	2.398
Cross-transplanted mangrove coral	1.000	1.000	0.354	-

Pairwise comparisons of Time points				
	Before	**T20**	**T44**	**One year**
Before	-	1.575	1.267	4.569
T20	0.738	-	2.138	8.587
T44	1.000	0.408	-	3.602
One year	< 0.05	< 0.01	0.072	-

Significant comparisons shown in bold. Pairwise comparisons between source × transplantations and between time points: upper values are model F-values, lower values are *p*-values.



Supplementary figure 3.3. Percentage composition of Hahellaceae taxa hosted by *Porites lutea* before and after reciprocal translocation between reef and mangrove habitats.

Chapter 4: Coral microbiomes are highly sensitive to active interventions: bacterial communities respond rapidly to antibiotic treatment and translocation

Abstract

Reef-building corals are running out of time to adapt to the imminent unfavourable conditions presented by anthropogenically-caused climate change. However, there are some corals already surviving under the warmer and more acidified conditions predicted for the next century, in marginal coral habitats such as mangroves. Changes in the communities of coral-associated microorganisms have been proposed as a potential means of rapid adaptation to new environmental conditions, but there is little evidence to support this hypothesis. This study aimed to partition the response of the coral holobiont to extreme conditions experienced in mangrove habitat, through antibiotic treatment and reciprocal translocations of corals between contrasting reef and mangrove habitats within the Wakatobi Marine National Park, Indonesia. Three coral species were studied; *Porites lutea* from both reef and mangrove habitat, *Goniastrea edwardsi* from reef habitat, and *Dipsastraea* cf. *pallida* from the mangroves. Housing corals for 36 hours prior to translocation, was found to significantly disrupt the coral microbiome, regardless of antibiotic treatment or not. Bacterial loading of corals, ascertained by quantitative PCR, increased significantly following incubation without antibiotics, while antibiotic treatment prevented rapid increases in bacterial abundance. Next-generation sequencing of bacterial 16S rRNA gene amplicons revealed that natural differences in the coral microbiome, before treatment or translocation, were driven by coral host species and habitat. A potentially novel coral-bacteria symbiosis was discovered

between *Dipsastraea* cf. *pallida* and an unidentified spirochaete. Following treatment and translocation, coral-associated bacterial communities shifted rapidly (within 96 hours), highlighting their susceptibility to disturbance as opposed to their potential for rapid holobiont adaptation. Furthermore, corals were found to be locally adapted, which resulted in a survivability trade-off when translocated to a new habitat for a year. These findings suggest that active interventions involving microbiome manipulation and translocation of corals might not be viable options for coral conservation.

4.1. Introduction

Scleractinian corals are ecosystem engineers which build vast calcium carbonate reefs, covering approximately 0.2% of the world's ocean and harbouring between a quarter and a third of known marine species (Reaka-Kudla, 2001). Coral reefs provide food and income to over half a billion people worldwide (UNEP, 2004; Wilkinson, 2004; Burke *et al.*, 2011). Therefore, the degradation of coral reefs represents a threat to global biodiversity, and the associated ecosystem services, valued at over US$352 000 $ha^{-1}yr^{-1}$ in 2011 (up from US$8 384 $ha^{-1}yr^{-1}$ in 1997; Costanza *et al.*, 1997, 2014).

The future of coral reefs is becoming ever-more uncertain due to multiple, compounding local and global threats, including habitat destruction, over-fishing, and pollution, on top of marine heatwaves, global warming, and ocean acidification (Hoegh-Guldberg *et al.*, 2007; Veron *et al.*, 2009). There is a real concern that as long-lived sessile organisms, corals will not be able to adapt and keep pace with the rapidly changing climate and accompanying more frequent and extreme marine heatwaves.

Unlike mobile taxa, such as butterflies and birds, which are able to quickly shift their ranges in response to changing climate (Parmesan & Yohe, 2003), corals remain cemented to the seabed, at the mercy of prevailing environmental conditions. Darwinian adaptation occurs over many generations, and thus over timescales much greater than the rapid environmental

change current corals face; there are predictions that 75% of coral reefs will be highly threatened by 2050 (Burke *et al.*, 2011). Other mechanisms by which corals could more rapidly adapt or acclimatise to new environmental conditions include 1) inheritable epigenetic changes in the coral genome, 2) acclimatisation or phenotypic plasticity, 3) algal symbiont switching or shuffling, and 4) changes to the composition of the coral microbiome. Transgenerational epigenetic changes to the coral host genome could potentially contribute to adaptation across just two generations. That is, changes in DNA methylation (the reversible addition of a methyl group to cytosine residues in DNA) accrued due to environmental conditions experienced by the parent coral colony, can be passed down to the offspring, to change gene activity (Liew *et al.*, 2018, 2020). Extensive DNA methylation was previously found across the genome of the coral, *Stylophora pistillata*, when chronically stressed with low-pH conditions (Liew *et al.*, 2018), and has since been found to be vertically transmitted from parent colony to sperm in the brain coral *Platygyra daedalea* (Liew *et al.*, 2020). Furthermore, heating experiments on larvae of these corals revealed survivability correlated with the methylation of certain stress- and growth-related genes (Liew *et al.*, 2020).

Acclimatisation to new conditions occurs within the lifetime of an organism, without any lasting genetic changes, and depends upon their phenotypic plasticity. The breadth of phenotypic responses available to the coral host in the face of environmental change is ultimately governed by its genotype (Coles & Brown, 2003a), and can take place slowly over seasons and years, or more quickly in the case of heat-hardening (Brown & Cossins, 2011). Cores from long-lived colonies of *Porites* have shown that high density skeletal ‘stress bands’, indicative of past bleaching, became less common following successive bleaching events, despite increasing frequency of high sea surface temperatures (DeCarlo *et al.*, 2019). This suggests that the coral colonies increased their thermal tolerance over the years through acclimatisation to past marine heatwaves. At the other end of the scale, *Acropora millepora* was shown to acquire elevated thermal tolerance following only ten days of heat-

hardening to experimentally controlled temperatures (i.e. acclimation; Bellantuono *et al.*, 2011).

As meta-organisms, corals form dynamic relationships with a multitude of microorganisms, including endosymbiotic algae of the family Symbiodiniaceae, bacteria, archaea, fungi, protists, and viruses (Knowlton & Rohwer, 2003; Bosch & McFall-Ngai, 2011). The combined genomes of a coral host and its respective microbial symbionts have been termed the coral hologenome, and are suggested, together, to define the phenotype and adaptive capacity of the coral holobiont (Zilber-Rosenberg & Rosenberg, 2008). Changes to the composition of a coral holobiont through reorganisation of its associated microbial communities can influence the coral's phenotypic traits, thereby influencing its ability to survive, and its ecological success. Coral-associated microbial community changes have been reported to take place over the course of months (Berkelmans & van Oppen, 2006; Ziegler *et al.*, 2017, 2019), days (Garren *et al.*, 2009), or even hours (Sweet *et al.*, 2011b), thereby representing a potential rapid intermediate means of adaptation.

Symbiodiniaceae were the first microbial partners of coral to be examined for their fast acclimatisation/adaptation potential. The adaptive bleaching hypothesis posed that corals could switch symbionts for more hardy genera (then known as clades, or types), after bleaching, to survive future environmental extremes (Buddemeier & Fautin, 1993; Buddemeier *et al.*, 2003). Studies have since shown that corals can switch algal symbionts or shuffle abundances of existing ones without the prerequisite of bleaching (Berkelmans & van Oppen, 2006; Reich *et al.*, 2017). Corals were shown to be capable of acquiring increased thermal tolerance as a direct result of a change in dominant symbiont type, after *Acropora millepora* translocated to a hotter reef gained approximately 1-1.5°C thermal tolerance, ascertained by heating experiment, compared with conspecific translocated corals which did not change symbiont composition (Berkelmans & van Oppen, 2006).

Attention is now turning to other coral-associated microorganisms for their adaptive potential (Voolstra & Ziegler, 2020). The coral probiotic hypothesis was developed to explain the resistance of coral *Oculina patagonica* to bacterial bleaching caused by *Vibrio shiloi*, and asserts that the environment selects for the most advantageous coral-microbiome composition (Reshef *et al.*, 2006). While there is an abundance of research which details the many roles bacteria can play as part of the coral holobiont (Sharp & Ritchie, 2012; Krediet *et al.*, 2013; Bourne *et al.*, 2016), there is scant evidence for a bacterial role in coral acclimatisation or rapid adaptation to changing environmental conditions. One study conducted between the thermally distinct back reef pools of Ofu Island, American Samoa, provided some evidence that the thermal tolerance of the coral, *Acropora hyacinthus*, might be causally linked to the microbial community hosted (Ziegler *et al.*, 2017). Certain bacterial taxa linked with corals living in high-thermal-variation habitat predicted the coral host response to short-term heat stress, though this could have been due to naturally high temperatures acting in concert on both coral host and microbiome.

In order to better understand the complex relationships between coral host and microbiota, and microbial interactions within the coral microbiome, experimental studies have sought to compartmentalise the coral holobiont through manipulation of the various holobiont members. Such active interventions include the removal or reduction of bacteria with antibiotics (Sweet *et al.*, 2011b; Mills *et al.*, 2013; Glasl *et al.*, 2016). Applications of specific antibiotics have been used in a targeted manner to establish the causative agents of coral diseases (Sweet *et al.*, 2014; Smith *et al.*, 2015; Sweet & Bythell, 2015), and various antibiotic mixtures, or ‘cocktails’, have been employed as a tool to ascertain the importance of the coral-associated bacterial community under varying experimental scenarios. For example, the importance of the bacterial community for coral thermal tolerance was investigated by administering a mixture of broad- and narrow-spectrum antibiotics, including ampicillin, streptomycin, ciprofloxacin and naladixic acid, to *Pocillopora damicornis* before heat-ramping (Gilbert *et al.*, 2012). The study concluded that an intact bacterial community

was indeed important in allowing coral to withstand heat stress. In addition, *Acropora muricata* and *Porites astreoides* have been subjected to antibiotic treatments in order to study the re-establishment of their bacterial communities *in situ* following disturbance; with bacterial reorganisation taking place in the order of hours to days (Sweet *et al.*, 2011b; Glasl *et al.*, 2016). More recently, the threat of a new stony coral tissue loss disease (SCTLD), which is rapidly spreading through the Florida Reef Tract, has reinvigorated research on antibiotics, and a topical paste containing the antibiotic, amoxicillin, already shows promise for halting the progression of disease lesions (Aeby *et al.*, 2019; Neely *et al.*, 2020).

Another experimental intervention which has been used to partition the coral holobiont involves deliberate chemical bleaching with menthol (Wang *et al.*, 2012a; Matthews *et al.*, 2016). The production of aposymbiotic corals (and coral model-organism, *Aiptasia* anemone) permits a multitude of experiments, such as partitioning the contribution to metabolic processes (Hawkins *et al.*, 2016), and re-inoculation with different genera or strains of Symbiodiniaceae to improve thermotolerance (Gabay *et al.*, 2019).

Experimental expulsion and exchange of coral-associated microbes could aid our understanding of natural processes such as horizontal transmission of microorganisms, but such manipulations could also provide avenues to explore accelerated rapid adaptation. Several avenues of active intervention are already being explored as a potential last resort for coral conservation. These include inoculation of probiotic bacteria or ‘beneficial microorganisms for corals’ (BMCs; Peixoto *et al.*, 2017), and experimental evolution or selective breeding of stress-tolerant symbionts (Chakravarti *et al.*, 2017; Chakravarti & van Oppen, 2018). Such interventions require much further understanding and testing before they could be considered as management options (Sweet *et al.*, 2017a).

Therefore, this study aimed to contribute to the growing body of knowledge regarding coral microbiome dynamics. It is the first to attempt to partition the coral holobiont in response to marginal mangrove habitat, through antibiotic treatment of corals before reef to mangrove

reciprocal translocations. Marginal habitats are environments which house corals outside of their classically perceived environmental optima, or close to their environmental limits (Kleypas *et al.*, 1999). The following hypotheses were addressed through a clonally replicated translocation experiment within Wakatobi Marine National Park, Indonesia: 1) If the microbiome is important in influencing the adaptive capacity of a coral holobiont then the composition of the microbiome should be specific to the local environment; 2) If the microbiome is environmentally-regulated then conspecific corals transplanted into and out of marginal-mangroves should reorganise their microbiome to match native holobionts, 3) If the holobiont community structure is dependent on environment then reduction of the native microbiota should result in re-colonisation from the local environment; 4) If coral holobionts are adapted to their local environment, this comes at the cost of the ability to survive in other environments; 5) If corals are able to acclimatise to new thermal regimes, then their thermal performance should change to suit the prevailing conditions.

4.2. Methods

4.2.1. Site characterisation

Coral collection sites were located within the Wakatobi Marine National Park, Southeast Sulawesi, Indonesia (Fig. 2.1). The fore-reef site, considered to have optimal conditions for coral survival, was situated off the southwest coast of Hoga Island, adjacent to the fringing reef crest, at a site known locally as ‘Buoy 2’ (5° 28' 31.2" S, 123° 45' 32.0" E). The mangrove site, considered to be a marginal habitat for coral to live in (Kleypas et al., 1999), was within a mangrove system characterised by *Rhizophora stylosa* trees, located at the northern coast of Kaledupa Island and known locally as ‘Langira’ (5° 28' 41.1" S, 123° 43' 17.4" E).

To characterise the environmental conditions of Buoy 2 and Langira, temperature and light were recorded using HOBO Pendant® Temperature/Light 64K Data Loggers (Model UA-

002-64, ONSET, USA). Water temperature was measured at 15-minute intervals for a year, between June 2017 – June 2018. Loggers were also deployed at 1 m depth intervals to calculate the light attenuation coefficient (K_d) and therefore turbidity of both sites.

Nutrient loading (DOC, POC, TN, TP) was assessed during the dry season (June – August 2018 in Indonesia) by taking triplicate 1.5L water samples at four high tides, each separated by a week, from both habitats. Water samples were syringe-filtered through pre-combusted (450°C for 4 h) 0.7 µm GF/F filters to separate dissolved and particulate fractions. Dissolved carbon (non-purgeable organic carbon and inorganic carbon) and total dissolved nitrogen were analysed on a Formacs TOC auto-analyser (Skalar).

Due to the low density of coral colonies in the mangrove habitat, belt transects of 12 m^2 (6 m x 2 m) (n = 3) were conducted with photoquadrats at both Buoy 2 reef and Langira mangrove, to capture coral density accurately. Corals were identified to genus level and substrate cover was estimated using Coral Point Count with Excel extensions (CPCe) software.

4.2.2. Experimental design

In June 2017, five colonies of each coral species (*Porites lutea*, *Goniastrea edwardsi*, and *Dipsastraea* cf. *pallida*) were collected from each habitat (Buoy 2 reef and Langira mangrove). Each coral colony was sampled before being fragmented into four. Each fragment from each colony was then assigned to be treated for 36 h with or without antibiotics, before transplantation into the same habitat (back-transplantation), or into a new habitat (cross-transplantation; Fig. 4.1). Fragmentation permitted a clonally replicated experimental design to control for the effect of coral genotype across treatments and translocations. Antibiotic-treated coral fragments were incubated for 36 hours in a mixture of broad spectrum antibiotics (Ampicillin 100 µg ml^{-1}, Streptomycin 100 µg ml^{-1}, Nalidixic acid 100 µg ml^{-1}), with water changes every 12 hours, before either back-transplantation to their

native habitat or cross-transplantation to the contrasting habitat. Non-treated coral fragments were subject to the same 36 h incubation procedure with 12 h water changes of 0.2µm filtered seawater. The reciprocal translocation followed a fully factorial experimental design. Coral host species (*Porites lutea*: family Poritidae, *Goniastrea edwardsi*: family Merulinidae, and *Dipsastraea* cf. *pallida*: family Merulinidae) were assessed separately or treated as a random factor in the experiment. Source habitat (mangrove vs. reef), antibiotic treatment (with or without antibiotics), and sampled habitat (mangrove vs. reef) were treated as fixed factors (Table 4.1).

Table 4.1. Fully factorial experimental design for translocation of *Porites lutea* and two merulinid corals between mangrove and reef environments in the Wakatobi Marine National Park, Indonesia. Antibiotic treated corals highlighted in grey.

		Origin habitat			
		Mangrove		Reef	
Transplant habitat	Mangrove	*Porites* (*n* = 5) *Dipsastraea* (*n* = 5)	*Porites* (*n* = 5) *Dipsastraea* (*n* = 5)	*Porites* (*n* = 5) *Goniastrea* (*n* = 5)	*Porites* (*n* = 5) *Goniastrea* (*n* = 5)
	Reef	*Porites* (*n* = 5) *Dipsastraea* (*n* = 5)	*Porites* (*n* = 5) *Dipsastraea* (*n* = 5)	*Porites* (*n* = 5) *Goniastrea* (*n* = 5)	*Porites* (*n* = 5) *Goniastrea* (*n* = 5)

To determine whether coral-associated microbial community compositions changed or reassembled following antibiotic treatment and translocation, corals were sampled before fragmentation (native), then immediately after 36 h antibiotic or seawater treatment (T0), and 4 days after reciprocal translocation (T96). Small coral tissue samples (< 2cm) were preserved in RNAlater (Ambion Inc.) for subsequent coral host identification, and enumeration and characterisation of microbial symbionts, by direct sequencing, quantitative real-time PCR, and multi-marker amplicon sequencing, respectively.

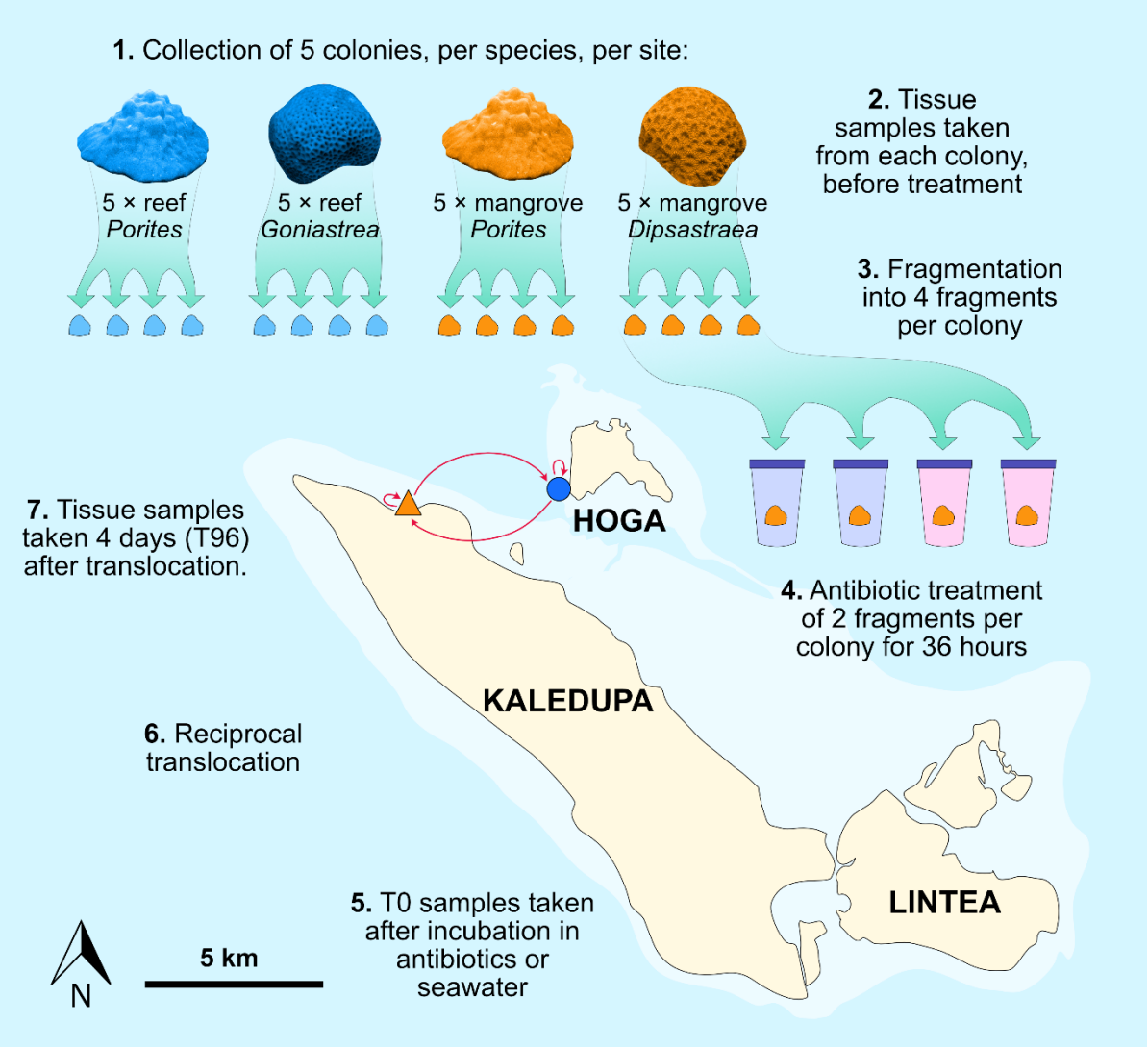


Figure 4.1. Schematic design of reciprocal translocation. Five coral colonies of each species at each site were collected and fragmented into four fragments per colony to allow for fully factorial antibiotic treatment and reciprocal translocation (total coral fragments = 80). Red arrows show back-transplantation and cross-transplantation of coral fragments from *Porites lutea*, *Goniastrea edwardsi*, and *Dipsastraea* cf. *pallida* between reef (blue circle) and mangrove (orange triangle) sites within the Wakatobi Marine National Park. GPS locations for Reef site 'Buoy 2': 5° 28' 31.2" S, 123° 45' 32.0" E, Mangrove site 'Langira': 5° 28' 41.1" S, 123° 43' 17.4" E.

4.2.3. Species identification of coral hosts

Coral hosts were identified by sequencing the eukaryotic gene region encompassing part of the 18S ribosomal RNA gene, the entire internal transcribed spacer 1 region (ITS1), the 5.8S ribosomal RNA gene, the internal transcribed spacer 2 region (ITS2), and part of the 28S ribosomal RNA gene. The phylogenetic marker region was targeted during PCR amplification using the coral-specific primer A18S: GATCGAACGGTTTAGTGAGG and

universal primer ITS4: TCCTCCGCTTATTGATATGC (Takabayashi *et al.*, 1998a; Huang *et al.*, 2011). Reaction mixtures of 50 µl were prepared with 25 µl 2x AppTaq RedMix (Appleton Woods), 2 µl of forward primer, 2 µl of reverse primer, 3 µl 1% BSA, 16 µl H_2O and 2 µl template DNA. Amplification conditions for the coral ITS PCR included an initial denaturation (95˚C for 3 min), followed by 35 cycles of: denaturing (95˚C for 15 sec), annealing (55˚C for 30 sec), extending (72˚C for 45 sec), then a final extension (72˚C for 7 min) and hold (4˚C) (Takabayashi *et al.*, 1998b). The PCR products were cleaned of primer-dimers using GenElute PCR Clean-Up Kit (Sigma Aldrich) and checked for confirmation of a single product by electrophoresis on 1.2% agarose gel. Direct (Sanger) sequencing of the partial 18S-ITS1-5.8S-ITS2-partial 28S gene region was performed via TubeSeq service by Eurofins Genomics UK (Eurofins Scientific).

4.2.4. Quantitative real-time PCR (qPCR)

Abundance of bacteria, Symbiodiniaceae, and Archaea were measured by quantitative real-time PCR (qPCR), as previously detailed in Chapter 3, on a C1000 Touch CFX384 Thermal Cycler (Bio-Rad) using SYBR-Green fluorophore. Briefly, reactions were performed in 10 µl volumes, containing: 5 µl of SensiFAST SYBR No-ROX (Bioline) mastermix reagent, 0.2 µl of each 10 µM primer (Table 3.1), 0.6 µl 1% BSA, 3 µl H_2O and 1 µl of template DNA. Cycling conditions were: 1 cycle of initial denaturation at 95°C for 3 min; 40 cycles of amplification consisting of 95°C for 5 s and 60°C for 30 s, then a final denaturation of 95°C for 5 s, followed by a final cycle of temperature ramping from 65°C to 95°C at 0.5°C per 5 s increments, for melting temperature curve analysis.

Each qPCR assay was internally calibrated against an environmentally relevant standard curve to produce copy number abundance estimations for *Porites* bacterial 16S rRNA (E = 90.7%, R^2 = 0.998, Slope = -3.566, y = 36.894), merulinid bacterial 16S rRNA (E = 91.4%, R^2 = 0.999, slope = -3.545, y = 36.444), *Porites* archaeal 16S rRNA (E = 72.7%, R^2 = 0.993,

Slope = -4.215, y = 38.563), merulinid archaeal 16S rRNA (E = 73.5, R^2 = 0.999, Slope = -4.179, y = 38.286), *Porites* Symbiodiniaceae ITS2 (E = 94.3%, R^2 = 0.998, slope = -3.466, y = 37.272), and merulinid Symbiodiniaceae ITS2 (E = 93.3%, R^2 = 0.998 , slope = -3.494, y = 37.000) regions.

4.2.5. Amplicon sequencing library preparation

Amplicon sequencing was carried out as detailed in Chapter 3. Briefly, the bacterial 16S rRNA gene and Symbiodiniaceae ITS2 region were amplified using primers 784F/1061R (Andersson *et al.*, 2008) and SYM_VAR (Hume *et al.*, 2018), respectively, with the addition of a MiSeq overhang (underlined) e.g. 784F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGATTAGATACCCTGGTA,

1061R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACACRRCACGAGCTGACGAC. Reaction mixtures of 25 µl were prepared with 12.5 µl 2x AppTaq RedMix (Appleton Woods), 0.5 µl of forward primer, 0.5 µl of reverse primer (Table 3.1), 1.5 µl 1% BSA, 9 µl H_2O and 1 µl template DNA. Amplification conditions for the bacterial PCR included an initial denaturation (95˚C for 3 min), followed by 27 cycles of: denaturing (95˚C for 15 sec), annealing (55˚C for 15 sec), extending (72˚C for 30 sec), then a final extension (72˚C for 7 min) and hold (4˚C). The PCR products were subsequently cleaned with Bioline JetSeq Clean solid phase reversible immobilisation (SPRI) beads (Scientific Laboratory Supplies), indexed over 8 PCR cycles with Nextera XT indexes (Illumina), and cleaned again with JetSeq SPRI beads. Each amplicon was quantified in triplicate, using PicoGreen dye (Quant-iT™ PicoGreen™ dsDNA Assay Kit, ThermoFisher Scientific), in 384-well plate format, on a plate reader (FLUOstar Omega Microplate Reader, BMG LabTech), before being pooled in equimolar ratios. Resulting gene libraries were pooled at a ratio of 4:1, 16S rRNA gene: ITS2, respectively, and cleaned using GenElute PCR Clean-Up Kit (Sigma Aldrich) to ensure no carry-over of SPRI beads. Sequencing was performed at 6pM

concentration with 17% phiX control, on the Illumina MiSeq platform, using a 600-cycle MiSeq Reagent Kit v3 (Illumina) to yield 2 × 300 bp overlapping paired-end reads. Negative mock DNA extractions and negative PCR controls were sequenced alongside samples to check for contamination. The resulting cluster density was 567K/mm^2.

4.2.6. Bioinformatics

The bacterial 16S rRNA gene amplicon library was processed following (Dumbrell *et al.*, 2017), as detailed in Chapter 3. Sequence reads were trimmed to 200 bases, before being quality trimmed using Sickle (Joshi & Fass, 2011), error corrected in SPAdes (Bankevich *et al.*, 2012) using the BayesHammer algorithm (Nikolenko *et al.*, 2013), and pair-end aligned with a minimum overlap of 15 bp with PEAR (Zhang *et al.*, 2014) within PANDASeq (Masella *et al.*, 2012). Pair-end aligned sequences shorter than 180 bp were removed. Sequences were then de-replicated, sorted by their abundance, and OTU centroids picked using VSEARCH at the 97% similarity level (Rognes *et al.*, 2016). All singleton OTUs were removed. Chimeric sequences were removed using reference-based chimera checking with UCHIME (Edgar *et al.*, 2011). Bacterial sequences were assigned to taxa using the RDP Classifier (a naïve Bayesian rRNA classifier; Wang *et al.*, 2007), with a 60% bootstrap confidence threshold.

The Symbiodiniaceae ITS2 amplicon library was processed remotely by SymPortal (Hume *et al.*, 2019), as also previously detailed in Chapter 3. Demultiplexed, paired sequences were submitted to SymPortal.org for quality control (Mothur 1.39.5; Schloss *et al.*, 2009, and BLAST + ; Camacho *et al.*, 2009) and minimum entropy decomposition (Eren *et al.*, 2015), before resolution of putative Symbiodiniaceae taxa (ITS2-type profiles) by defining intragenomic ITS2 sequence variants (DIVs).

4.2.7. Statistical analyses

All analyses were conducted using the statistical programming software `R`, version 3.6.1 (R Core Team, 2019). Microbial community analyses were conducted on data which had been filtered for non-target sequences, and rarefied to 8000 sequences per sample, using the `phyloseq` package within `R` (McMurdie & Holmes, 2013). The resulting dataset comprised 11313 unique OTUs from 180 coral and seawater samples. Alpha diversity metrics were calculated for the bacterial community of each sample using `phyloseq` (McMurdie & Holmes, 2013). Permutational multivariate analyses of variance (PERMANOVAs) were used to test whether bacterial communities were more dissimilar between coral host species, habitats, or treatments, than within them, at each sampling point, using 999 permutations with the function `*adonis*` in the R package `vegan` (Anderson, 2001; Oksanen *et al.*, 2019). These results were illustrated by non-metric multidimensional scaling (nMDS) ordination.

To identify bacterial taxa indicative of certain habitats or treatments, separate multivariate generalised linear models (MV-GLMs) were run for each coral host species with the R package `mvabund` (Wang *et al.*, 2012b). All samples were initially screened for bacterial taxa found to associate with particular coral host species. But since different coral species are known to react differently to microbiome disturbances (Ziegler *et al.*, 2019), samples from different coral host species were then separated for analysis independently. Operational Taxonomic Units (OTUs) were agglomerated by genus (except unclassified OTU1 which was kept separate), and models were specified with a negative binomial distribution to account for overdispersion – a common trait of microbial community data due to the high occurrence of zeroes. To investigate whether there were any particular OTUs of interest, by habitat or treatment, MV-GLMs were performed on a stringently filtered OTU table, with any OTUs which did not occur at least 5 times in at least 4 samples removed (taxa with such low abundance and prevalence were unlikely to be indicative of habitat or treatment).

Symbiont composition of corals four days after translocation (T96) was assessed following sequencing of the ITS2 region. Internal transcribed spacer region 2 (ITS2) sequence variants and the Symbiodiniaceae type profiles derived from the prevalence patterns of the DIVs (defining intragenomic sequence variants) were analysed separately. Average relative sequence abundances and type profile abundances were calculated by coral host species, and translocation, using phyloseq. According to the SymPortal framework, ITS2 sequences commonly found in the literature were assigned with their known names (e.g. C3, C15, or C15ai). Unclassified sequences were assigned a unique ID from the SymPortal database with the corresponding Symbiodiniaceae clade (e.g. 170815_C refers to a sequence with the unique ID 170815 from clade C, or *Cladocopium* genus). The Symbiodiniaceae type profile names assigned by SymPortal are informative: capitalised letters denote the algal clade or genus of that putative taxon and hyphens separate the component DIVs making up that profile, in decreasing order of abundance e.g. profile C15-C15bq refers to a genotype of *Cladocopium* genus, where the C15 sequence variant is most abundant, and C15bq sequence is next abundant. Symbiodiniaceae taxa characterised by co-majority abundances of component DIVs are denoted by a forward slash, e.g. C15/C15ed (Hume *et al.*, 2019).

4.2.8. Coral survival and thermal performance of transplants

In order to assess whether corals were locally adapted to reef or mangrove habitats, the transplanted corals were revisited one year after translocation, and their survival noted.

Three of the *Porites lutea* colonies at each habitat which had survived transplantation were chosen to assess their thermal performance. Thermal performance of *P. lutea* translocated from the thermally stable fore-reef to the temperature-extreme mangrove habitat for one year was compared with that of back-transplanted *P. lutea* which remained at the fore-reef for one year (n = 3). If *P. lutea* was able to acclimatise to the new thermal regime, its thermal performance should have changed to suit the mangrove environment. Thermal performance

curves were constructed using light and dark metabolic rates across a temperature range from 20°C to 38°C (using methods developed in Chapter 2 & Appendix I). Cardinal temperatures (optimum temperatures for productivity, T_{optP}, and respiration, T_{optR}) were extracted from fitted values of the best-fitting thermal response equations using the R package `temperatureresponse` (Low-Décarie *et al.*, 2017; Low-Decarie *et al.*, 2018).

4.3. Results

4.3.1. Environmental conditions

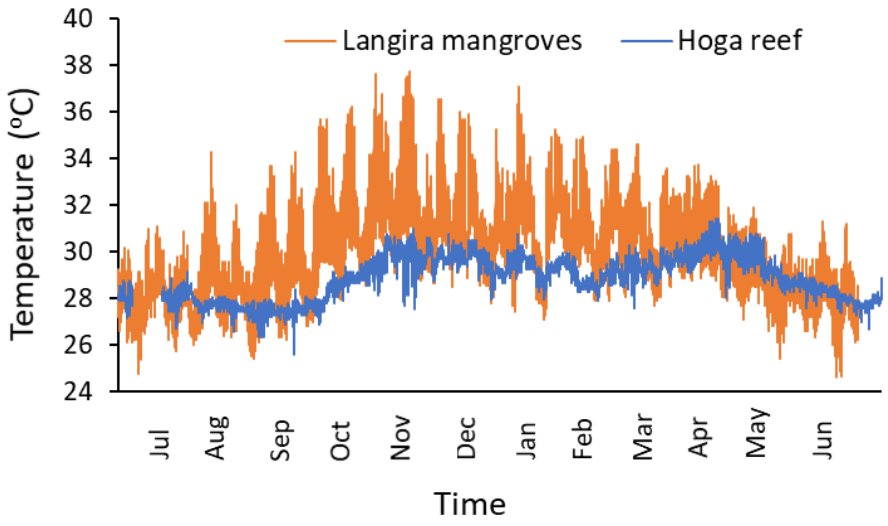


Figure 4.2. Time series of sea temperature from July 2017 - June 2018 for Hoga reef (blue) and Langira mangrove (orange).

Temperature was more variable in the mangrove habitat than on the reef, reaching temperatures in excess of 37°C (Fig. 4.2). Nutrient loading was consistently higher at Langira mangrove than at Hoga reef (Fig. 4.3).

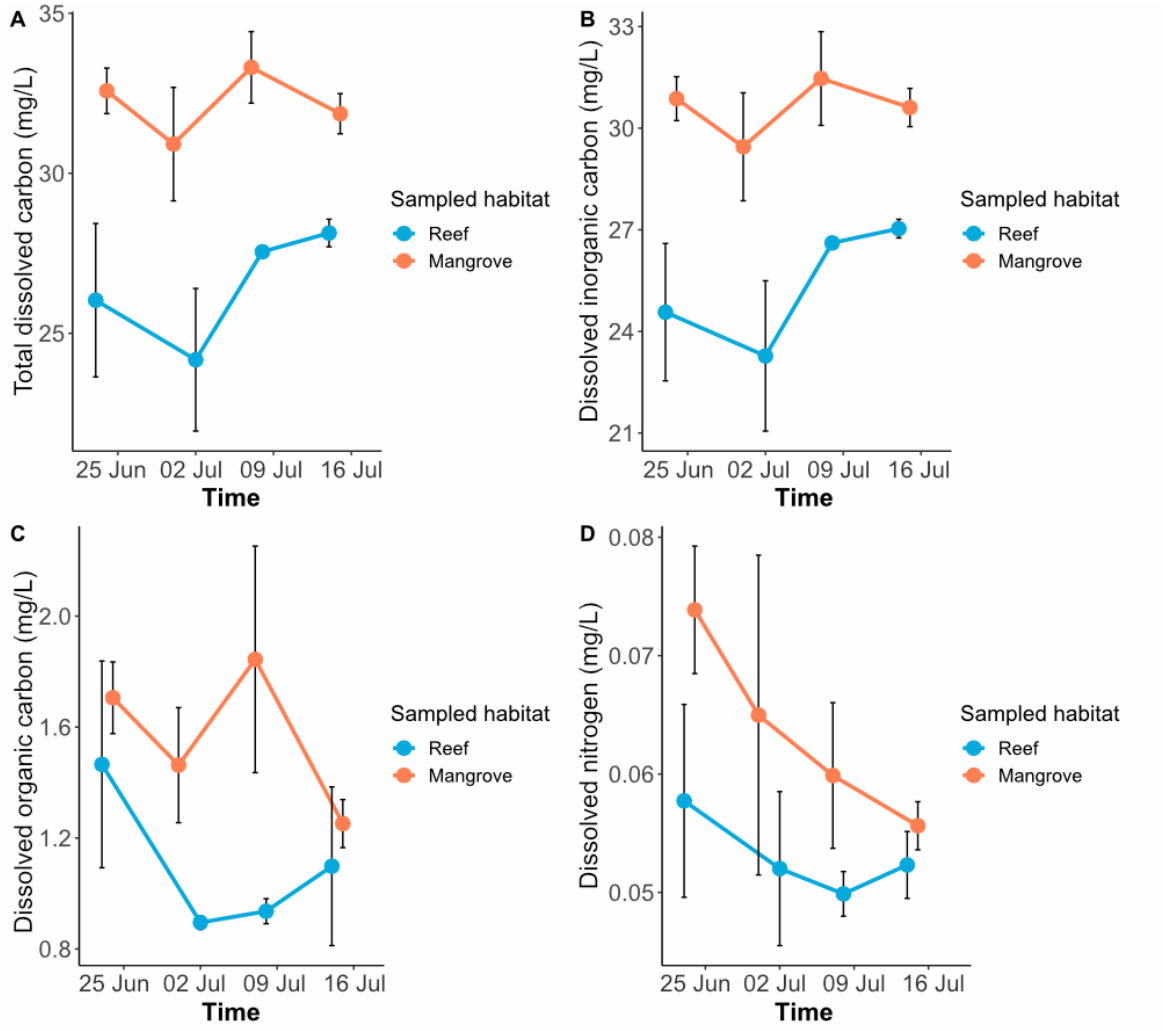


Figure 4.3. Nutrient loading of water from both Hoga reef (blue) and Langira mangrove (orange) sites, Wakatobi Marine National Park, Indonesia in June-July 2018. **A)** Total dissolved carbon; **B)** Dissolved inorganic carbon; **C)** Dissolved organic carbon; **D)** Total dissolved nitrogen.

4.3.2. Benthic characterisation

The surveyed area of Buoy 2 fore-reef hosted in excess of 30 different coral genera spanning all major growth forms but was dominated by branching *Porites* species such as *P. nigrescens* and *P. cylindrica.* The abiotic substrate was largely consolidated rubble. Langira mangrove had a drastically reduced diversity in terms of coral morphology and species richness compared with the nearby fore-reef. The vast majority of corals (> 95%) were massive or encrusting, except for free-living fungid corals, and newly settled recruits. Only five distinguishable hard coral taxa were found during mangrove surveys, in addition to some

unidentified Zoantharia. The scleractinians recorded in mangrove surveys were *Dipsastraea* cf. *pallida*, *Favites* cf. *bestae*/*pentagona, Porites lutea*, *Heliofungia actiniformis*, and recruits of *Pocillopora damicornis*. The coral cover in the mangrove was spatially heterogeneous with some areas lacking any corals. The remaining benthic cover comprised *Caulerpa* algae, *Enhalus* seagrass, and a mixture of coarse sand and fine organic silt on top of a hard calcium carbonate bed.

4.3.3. Species identification of coral hosts

Based on partial 18S-ITS1-5.8S-ITS2- partial 28S rRNA sequences, the mangrove merulinids belonged to clade XVII-B (Huang *et al.*, 2011, 2014), and were closely related to *Dipsastraea pallida* (formerly *Favia pallida*) and *Coelastrea aspera* (formerly *Goniastrea aspera*)(Fig. S4.1). Based on phenotype and skeletal morphology, the mangrove merulinids appeared most similar to *Coelastrea aspera* or the type taxon *Coelastrea tenuis* (for which no sequence is available; (Fig. S4.2).

4.3.4. Microbial abundance

Bacteria were by far the most abundant microorganism measured in seawater and coral (Fig. 4.4 & Fig. 4.5). Bacteria totalled 6.91×10^6 to 7.93×10^7 16S rRNA gene copies L^{-1} seawater from Buoy 2 fore-reef, and were present in higher concentrations in Langira mangrove, ranging from 1.39×10^7 to 1.43×10^8 16S rRNA gene copies L^{-1} seawater (Fig. 4.4). Symbiodiniaceae were found in relatively low abundances free-living in seawater (1.65×10^3 – 1.12×10^5 ITS2 copies L^{-1}; Fig. 4.4) compared with *in hospite* (6.06×10^6 – 2.07×10^{10} ITS2 copies g^{-1}; Fig. S4.3). Archaea were found in similar magnitude abundance (~ 10^4 – 10^5) in seawater (Fig. 4.4) and in corals (Fig. S4.3).

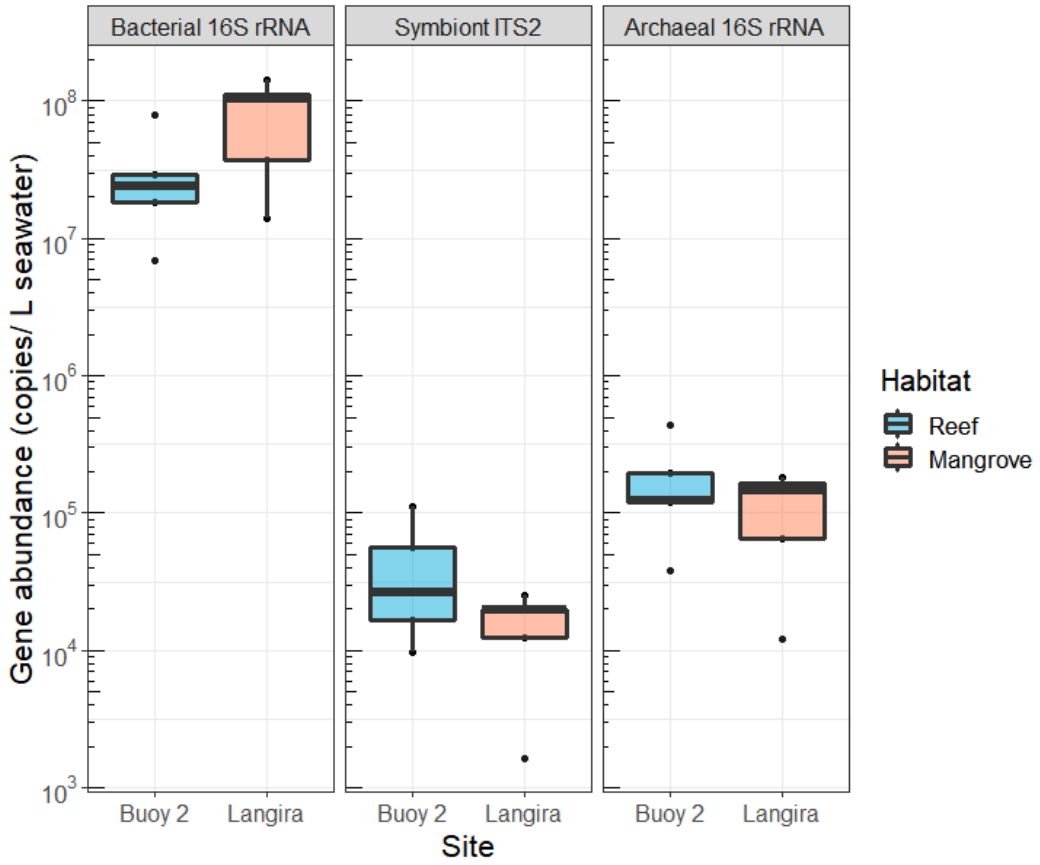


Figure 4.4. Microbial loading of seawater (bacterial 16S rRNA, Symbiodiniaceae ITS2, archaeal 16S rRNA gene copies per litre, ascertained by qPCR) from reef (Buoy 2) and mangrove (Langira) habitat. Boxplots represent median and interquartile range (*n* = 5 samples per habitat; blue = reef; orange = mangrove water), plotted on a logarithmic scale.

4.3.4.1. Effect of antibiotics on coral-associated bacterial abundance

Antibiotic treatment caused a highly significant reduction in coral-associated bacterial abundance, when compared with non-treated corals ($F_{(1, 126)} = 47.81$, $P < 0.001$). Immediately after treatment (T0), bacteria in reef *Porites* treated with antibiotics averaged $8.94 \pm 3.8 \times 10^7$ 16S rRNA copies g^{-1} coral tissue, while reef *Porites* incubated without antibiotics had $2.7 \pm 0.92 \times 10^8$ bacterial 16S rRNA gene copies g^{-1} coral tissue. Reef *Goniastrea* showed a similar pattern with $2.26 \pm 0.54 \times 10^7$ bacterial 16S rRNA gene copies g^{-1} immediately after treatment with antibiotics, compared to $5.06 \pm 2.51 \times 10^8$ 16S rRNA gene copies g^{-1} without antibiotics. The bacterial loading of *Porites* from the mangrove differed similarly after treatment with ($5.33 \pm 2.75 \times 10^7$) and without ($4.75 \pm 0.74 \times 10^8$)

antibiotics, as did *Dipsastraea* from the mangrove (with antibiotics: 9.31 ± 6.7 × 10^7, without antibiotics: 1.24 ± 1.14 × 10^9 16S rRNA copies g^{-1}).

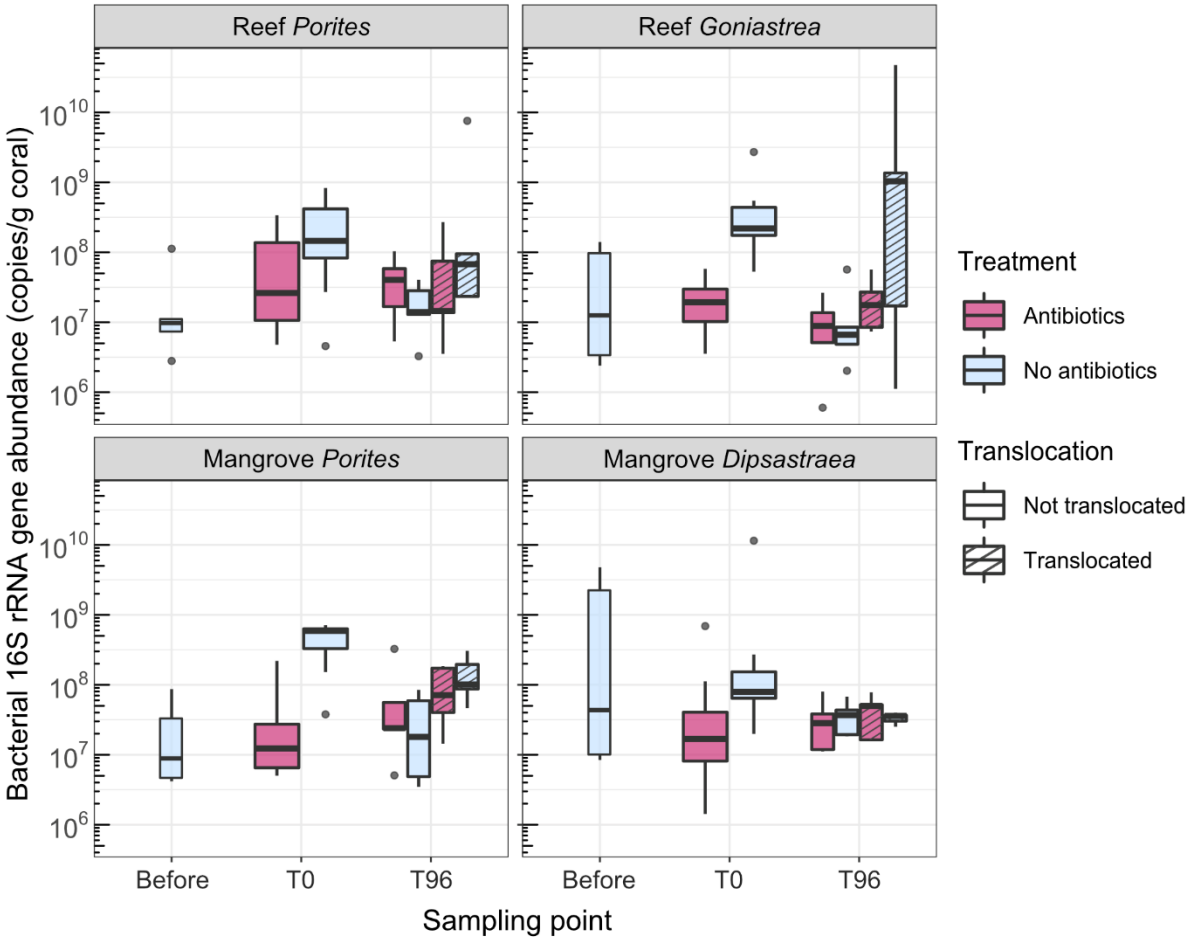


Figure 4.5. Bacterial loading (i.e. abundance of the bacterial 16S rRNA gene, ascertained by qPCR) of *Porites lutea, Goniastrea edwardsi,* and *Dipsastraea pallida* before treatment, immediately after 36h incubation in antibiotics or seawater (T0), and 96 hours i.e. 4 days after treatment and translocation (T96). Antibiotic treated corals shown in pink, corals incubated in seawater-only shown in light blue, translocated corals with striped pattern. Boxplots depict median and interquartile range. Before treatment (native to habitat) represent *n* = 5 coral colonies, T0: *n* = 10 coral fragments, T96: *n* = 5 coral fragments.

When comparing the natural bacterial loading of native coral colonies (before treatment) with that of colonies immediately after treatment (T0), with the exception of mangrove *Dipsastraea* colonies, there appears to have been a bacterial accumulation effect of incubating corals in a pot for 36h (regardless of antibiotics; Fig. 4.5). In other words, there was a significant increase in the average bacterial abundance hosted by *Porites,* and

Goniastrea, from before treatment to immediately after 36h incubation without antibiotics ($P < 0.05$).

Translocation had little to no effect on bacterial loading of corals ($F_{(3, 117)} = 0.62$, $P > 0.05$). But there was a significant effect of time ($F_{(1, 142)} = 4.36$, $P < 0.05$) and an interactive effect of treatment over time ($F_{(1, 126)} = 4.48$, $P < 0.05$). Coral-associated bacterial abundance generally decreased over 4 days (T96) following incubation without antibiotics, but remained at a similar level for corals treated with antibiotics.

4.3.5. Bacterial community composition

Next-generation sequencing of bacterial 16S rRNA gene amplicons produced sequences which clustered into 12,968 distinct Operational Taxonomic Units (OTUs) from 190 samples. After filtering of non-target taxa (Archaea, chloroplast, mitochondria sequences) and rarefaction to a depth of 8000 sequences per sample, there were 11,313 taxa from 180 samples.

4.3.5.1. Diversity of coral-associated bacterial communities

Coral-associated bacterial communities were naturally different in terms of diversity between coral host species and habitats (Fig. 4.6). Before treatment or translocation, *Porites lutea* from the mangrove hosted bacterial assemblages with higher OTU richness and evenness than those bacterial communities associated with *Porites* from the reef habitat. On the other hand, *Goniastrea* from the reef naturally hosted very even bacterial communities while the other merulinid coral, *Dipsastraea*, from the mangrove, hosted uneven assemblages consistently dominated by a single OTU (OTU 1; Fig. 4.8).

Antibiotic treatment reduced the bacterial diversity hosted by reef *Porites* and reef *Goniastrea* in terms of evenness, but where mangrove *Dipsastraea* naturally hosted uneven bacterial assemblages dominated by one OTU, antibiotic treatment resulted in an increase in

bacterial diversity, as did incubation for 36h without antibiotics (Fig. 4.6). Immediately after treatment, corals treated with antibiotics generally hosted less diverse bacterial assemblages than those not treated with antibiotics (Fig. 4.6), with the exception of mangrove *Porites*, whose bacterial diversity decreased due to domination by Alteromonadales and Vibrionales following incubation without antibiotics (Fig. 4.8).

Four days (96 hours) after treatment and translocation, antibiotic-treated corals generally had more diverse bacterial communities than non-antibiotic-treated corals. This pattern was more pronounced in the merulinid corals, *Goniastrea* and *Dipsastraea*. Translocation had no effect on the diversity of coral-associated bacterial communities.

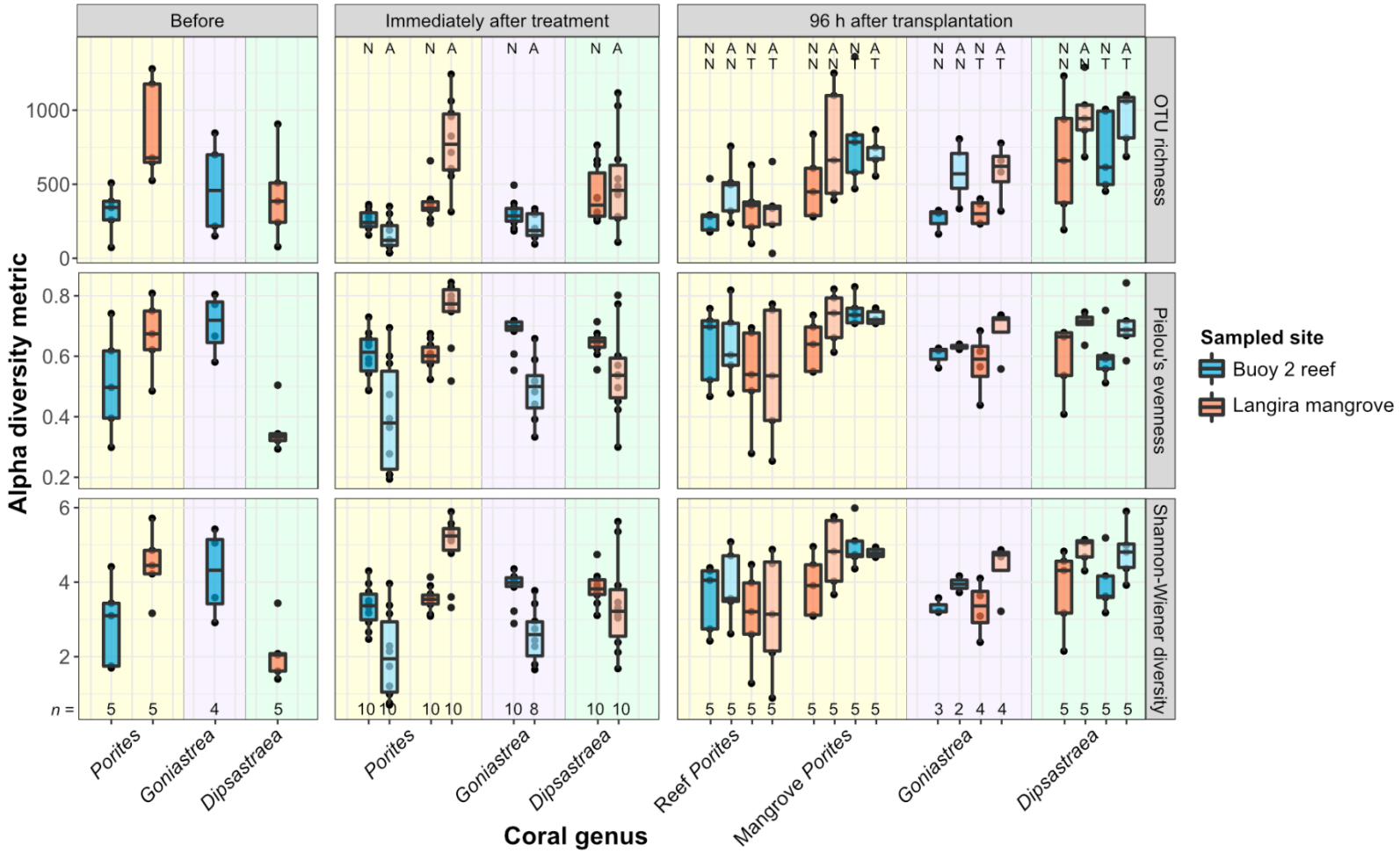


Figure 4.6. Alpha diversity metrics (OTU richness, Pielou's evenness, Shannon-Wiener diversity) of bacterial community associated with *Porites lutea* (yellow background), *Goniastrea edwardsi* (purple background), and *Dipsastraea pallida* (green background) sampled at Buoy 2 reef and Langira mangrove, before and after treatment and translocation (top facets show sampling point). Values are median and interquartile range, based on counts rarefied to 8000 reads per sample. Superscript denotes treatment (A: antibiotics, N: no antibiotics) and translocation (T: cross-transplanted, N: back-transplanted). Subscript denotes number of samples per group (*n*).

4.3.5.2. Dissimilarity of bacterial communities

Coral-associated bacterial communities were naturally dissimilar based on coral host species and habitat (Fig. 4.7 Before panel; Fig. S4.4 A & C; Table S4.1). The bacterial communities of all coral host-habitat combinations were naturally distinct, with the exception of *Goniastrea* compared with *Porites* from either habitat (Table S4.1). This was partly because *Goniastrea* samples exhibited greater within-group dispersion. Coral-associated bacterial communities of antibiotic treated corals were distinct from non-antibiotic-treated corals, immediately after treatment, but converged within 96 hours post-translocation (Fig. 4.7; Fig. S4.4 B). Bacterial communities were also different (i.e. dissimilar) based on habitat before translocation (T0; Fig. S4.4 C). While nMDS ordination of samples from all sampling points suggested that bacterial communities of corals translocated to a new environment were not dissimilar to coral back-transplanted within the original habitat (Fig. 4.7), separate PERMANOVA analyses within time points revealed fine-scale significant differences between sampled (destination habitats) four days after translocation (T96; Fig. S4.6; Table S4.1).

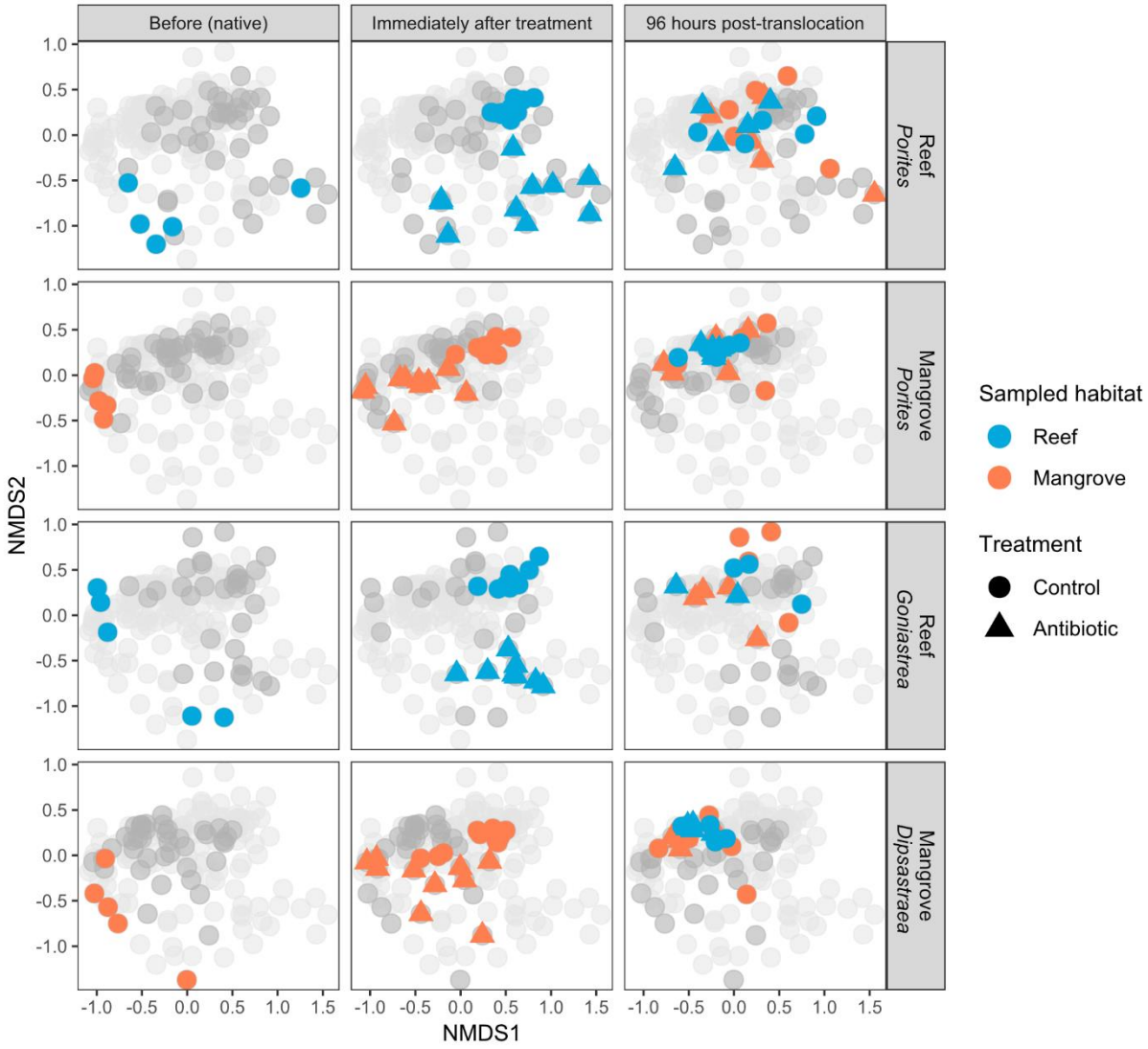


Figure 4.7. Non-metric Multi-Dimensional Scaling (nMDS) ordination of bacterial community composition based on Bray-Curtis dissimilarity (2D stress = 0.19). Bacterial community dissimilarity illustrated for *Porites lutea* from both reef and mangrove habitat, *Goniastrea edwardsi* from the reef, and *Dipsastraea* cf. *pallida* originally from the mangrove, before treatment (i.e. natural native composition), immediately after treatment, and 96 hours post-translocation. Symbols represent samples, symbol colours denote sampled site (blue: Buoy 2 fore-reef, orange: Langira mangrove), symbol shapes denote treatment (circle: seawater-only control, triangle: antibiotic treatment).

4.3.5.3. Coral-associated bacterial community composition

The coral-associated bacterial community of *Porites lutea* from the reef was naturally dominated by Oceanospirillales (49%), of which 28% were from the family Hahellaceae, which contains the known endosymbiont, *Endozoicomonas* (Fig. 4.8). *Porites lutea* from the mangrove, on the other hand, was dominated by Rhodobacterales (18%), green sulphur

bacteria (Chlorobiales; 3%), purple sulphur bacteria (Chromatiales; 4%), and Rhodospirillales (4.5%). Desulfobacterales also comprised 3% of the bacterial community of native mangrove *Porites*, and were found to have increased in relative abundance from < 1% to around 6% in *Porites* originally from the reef when translocated from reef to mangrove habitat. *Goniastrea* native to the reef habitat had a more even bacterial community composition (Fig. 4.6), consisting mainly of Cytophagales (13%), Rhodobacterales (6%), Spirochaetales (6%), Flavobacteriales (3%), Oceanospirillales (2%), Rhodospirillales (2%), and unclassified Alphaproteobacteria (16%). The natural bacterial community of *Dipsastraea,* native to the mangrove habitat, was dominated by a single bacterial phylotype (OTU 1; a putative Spirochaete based on closest cultured relative in the NCBI database; 88.22% sequence similarity with *Spirochaeta isovalerica;* NR_117137), comprising, on average, 47% of the total community.

Treatment for 36h without antibiotics caused a large increase in the relative abundance of Alteromonadales and Vibrionales in all coral species (Fig. 4.8). The increase in relative abundance of Alteromonadales was especially pronounced in *Porites* from the mangrove where Alteromonadales accounted, on average, for 40% of the bacterial community (Fig. 4.8). Taking the qPCR data into account, this equated to an estimated change in average absolute abundance of Alteromonadales from approximately 1.09×10^5 16S rRNA copies g^{-1} before treatment, to 1.91×10^8 16S rRNA copies g^{-1} after 36h incubation without antibiotics. Vibrionales increased from 1.5% to 27% of the bacterial community of reef *Porites*, following incubation without antibiotics, and from < 1% to 27% in mangrove *Porites*. In *Goniastrea,* Vibrionales increased from < 1% to 17%, and in *Dipsastraea,* from < 1% to 22% relative abundance of the bacterial community. Even with antibiotics, corals of all species incubated for 36h hosted an increased relative abundance of Vibrionales compared with before treatment, but to a much lesser extent than when incubated without antibiotics.

Four days after translocation, Flavobacteriales comprised a larger proportion of the coral-associated bacterial community for all coral species than at previous sampling points (Fig. 4.8). This was particularly pronounced in *Porites* back-transplanted within the reef (19%), or cross-transplanted from mangrove to reef (17%). Bacteroidales (particularly belonging to the genus *Marinifilum*) became more relatively abundant in both *Porites* (4%) and *Goniastrea* (11%), originally from Buoy 2 reef, following translocation to Langira mangrove.

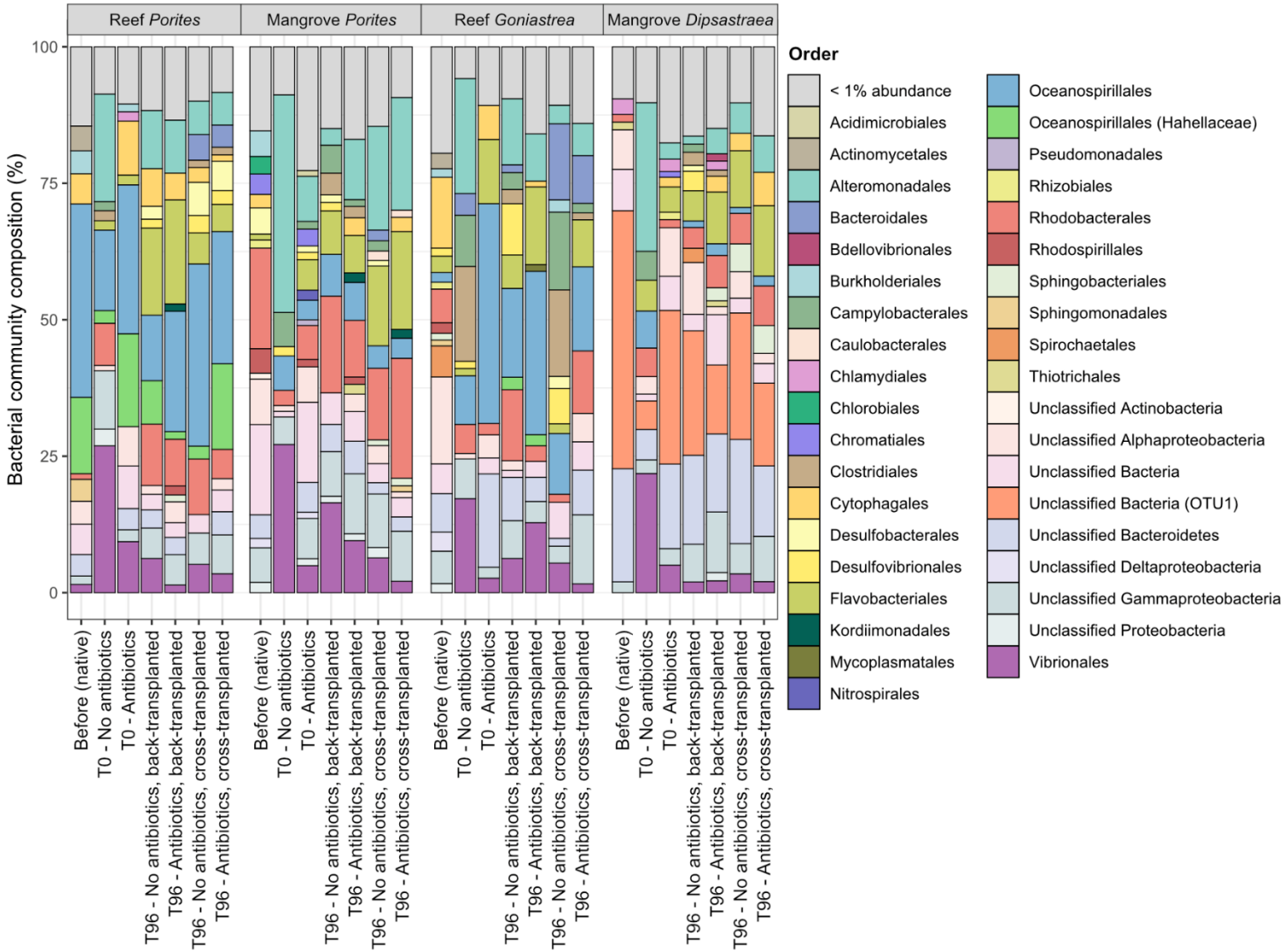


Figure 4.8. Average relative abundance (%) of bacterial orders (based on 16S rRNA gene sequences), associated with *Porites lutea, Goniastrea edwardsi*, and *Dipsastraea* cf. *pallida* treated, then reciprocally translocated between mangrove and reef habitat. X-axis shows treatment and sampling point. Colours represent the most abundant bacterial orders (> 1% mean abundance). Lower rank taxa of particular interest depicted separately in brackets. Remaining taxa are grouped as '< 1% abundance'.

4.3.5.4. Differentially abundant bacteria

Multivariate generalised linear models (MV-GLMs) revealed 46 genera which were significantly affected by either antibiotic treatment, habitat, or a treatment-habitat interaction in at least one of the coral host species (Table S4.2). There were more bacterial genera associated with incubation without antibiotics, than there were of corals treated with antibiotics. Antibiotic treatment was a statistically important predictor of the relative abundance of *Litoricola* (Oceanospirillales) and *Erythrobacter* (Sphingomonadales), which were found in greater relative abundance in antibiotic treated corals than corals incubated without antibiotics (Fig. 4.9). On the other hand, *Alteromonas*, *Marinobacterium*, *Pseudoalteromonas*, and *Thalassotalea* of the Alteromondales, and *Allomonas*, *Photobacterium*, and *Vibrio* of the Vibrionales made up more of their respective bacterial communities after incubation of corals without antibiotics (Fig. 4.9).

There were some bacterial genera which were affected by treatment and/or translocation when associated with one coral host, but not with another. *Ruegeria*, for example, became more prevalent in terms of relative abundance (and absolute abundance, based on 16S rRNA gene copies from qPCR) in *Porites* from the reef, at all time points after treatment without antibiotics. But this pattern was not observed in the other corals studied. Other bacterial genera, namely within the order Alteromonadales, consistently increased in relative (and absolute) abundance across all coral hosts following incubation without antibiotics.

There were relatively few bacterial genera for which habitat was a significant predictor across all sampling points, and antibiotic treatment (or lack of) generally resulted in larger effect sizes. However, *Arcobacter* (Campylobacterales) was significantly more abundant in corals sampled in Langira mangrove.

The unclassified Spirochaete, OTU1, was tightly linked to the mangrove-origin coral *Dipsastraea* cf. *pallida*, accounting for > 47% of the natural undisturbed bacterial community

(before treatment or translocation), and never comprising more than 0.1% of the bacterial community of the other corals studied. Incubation for 36h without antibiotics caused a shift in the bacterial community composition of *Dipsastraea* resulting in OTU1 accounting for only 5% of the bacterial community.

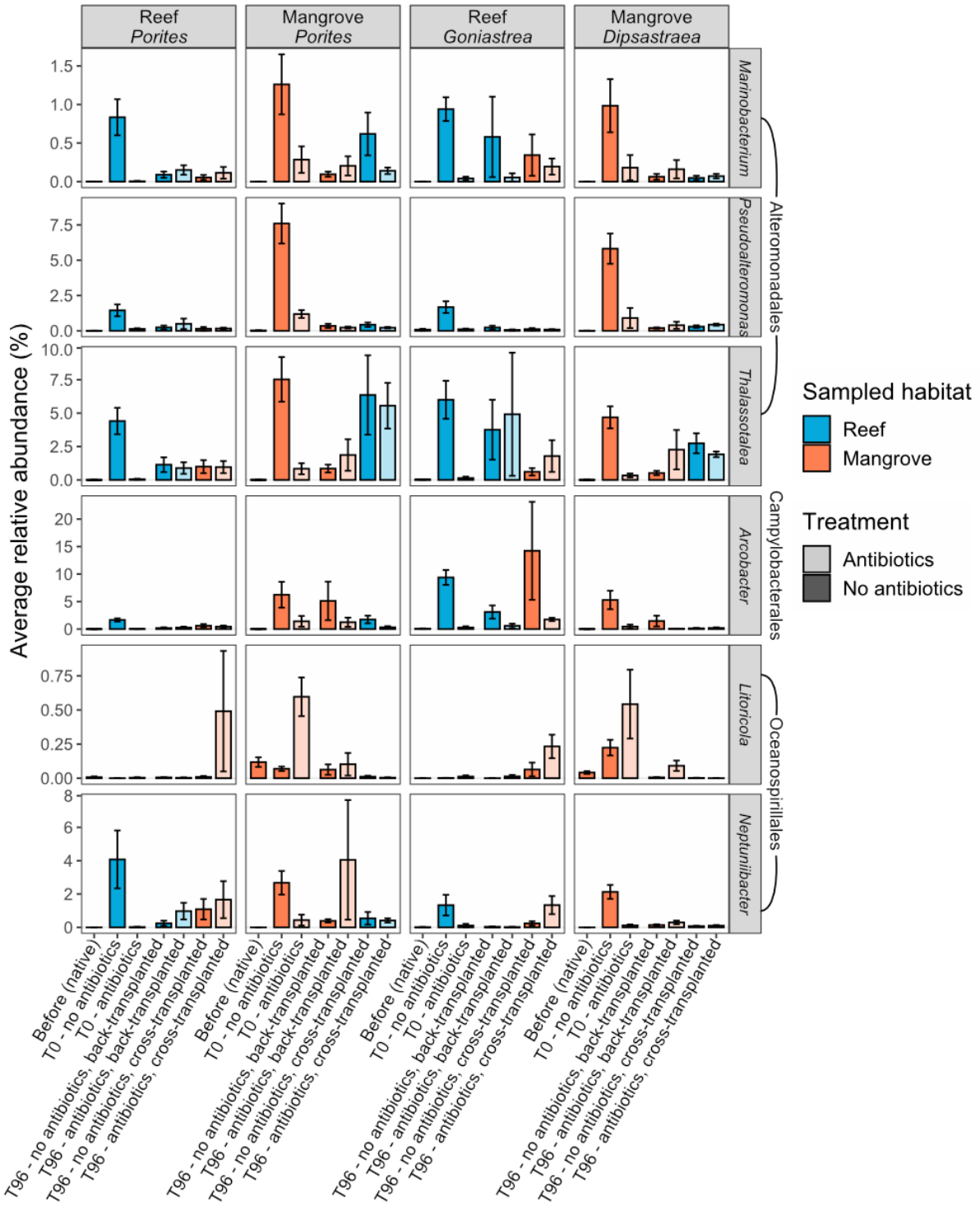


Figure 4.9. A) Average relative abundance (mean ± SE %) of coral-associated bacterial genera for which habitat or treatment were a statistically important predictor (determined by MV-GLM). Genera and corresponding Orders are shown to the right of the plot. Only genera whose average relative abundance was > 0.5% are shown, arranged alphabetically by Order.

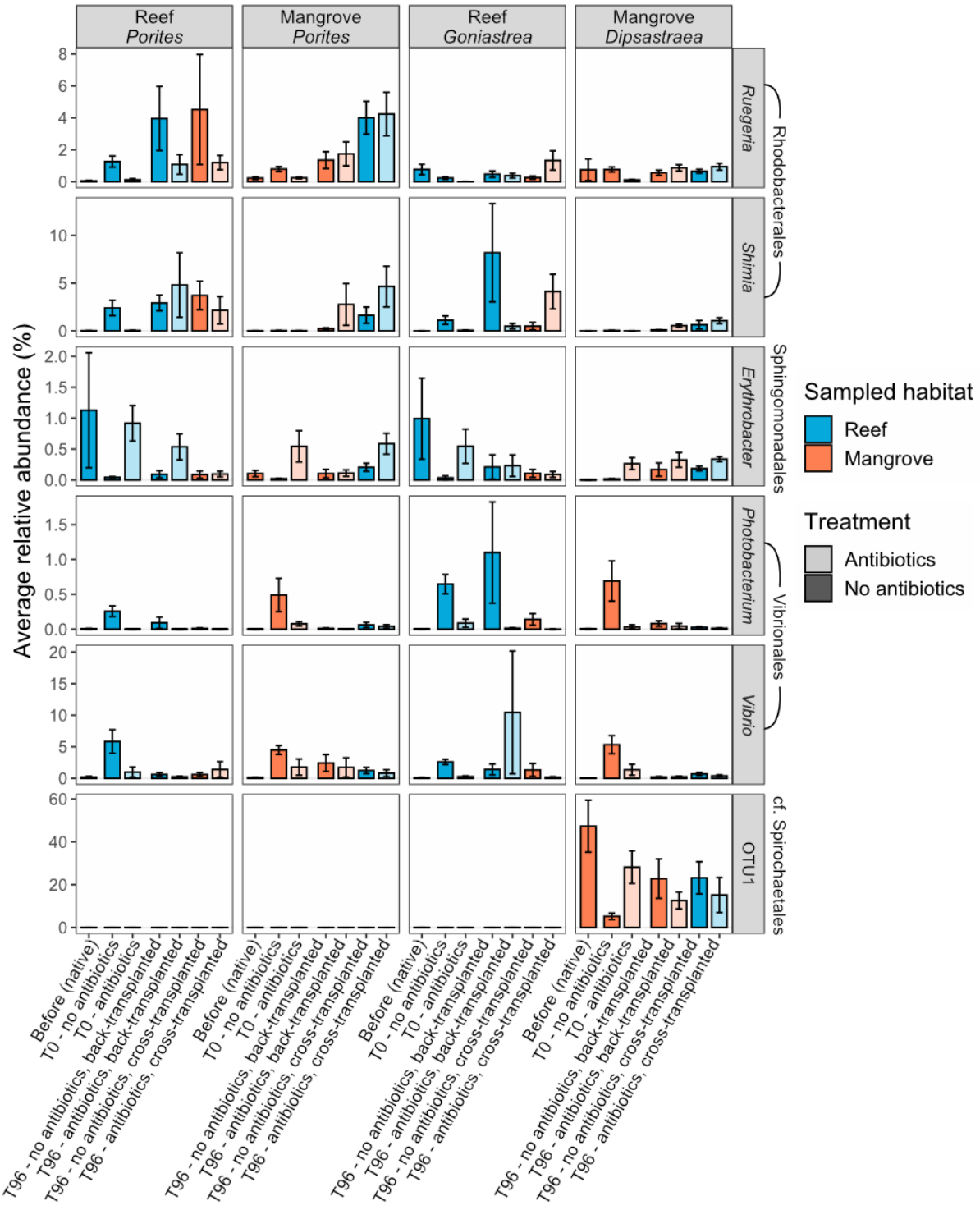


Figure 4.9. B) Average relative abundance (mean ± SE %) of coral-associated bacterial genera for which habitat or treatment were a statistically important predictor (determined by MV-GLM). Genera and corresponding Orders are shown to the right of the plot. Only genera whose average relative abundance was > 0.5% are shown, arranged alphabetically by Order.

4.3.6. Coral-algal symbiosis

Porites lutea from both habitats was dominated by *Cladocopium* endosymbionts of the sub-clade C15 (Fig. 4.10). Over 81% of symbiont ITS2 sequences from *Porites lutea* from the reef, and 88% from the mangrove, belonged to just one sequence variant (C15), with the remaining sequences comprised of rare ITS2 sequence variants (Fig. 4.10 top). The assignment of ITS2 sequence variants to putative Symbiodiniaceae taxa by SymPortal analysis revealed the possible presence of ten distinct ITS2 type profiles hosted across *Porites lutea.* SymPortal analysis indicated that different Symbiodiniaceae genotypes were hosted by corals from the reef versus the mangrove (Fig. 4.10 bottom). *Goniastrea edwardsi,* found only at Buoy 2 fore-reef, was also dominated by endosymbionts of the genus *Cladocopium*, but had ITS2 sequences annotated as C40, C3, and C115. SymPortal analysis assigned these sequence variants to one putative Symbiodiniaceae taxon with the type profile C40-C3-C115 (representing the component defining intragenomic ITS2 sequence variants, DIVs, in decreasing order of abundance). *Dipsastraea* cf. *pallida* from Langira mangrove also consistently hosted only one putative Symbiodiniaceae genotype of the genus *Durusdinium*: D1/D4-D1bo-D4c-D1bp-D1bn. The ITS2 sequences recovered from *Dipsastraea* were more evenly distributed between ITS2 sequence variants but were consistently hosted in such relative abundances across all *Dipsastraea* samples that SymPortal analysis deduced the sequences to derive from a single *Durusdinium* endosymbiont taxon. Symbiont identities remained the same for cross- and back-transplanted colonies of *Goniastrea* and *Dipsastraea,* four days after translocation. Symbiont communities were largely similar for cross- and back-transplanted colonies of *Porites lutea,* but remained distinct dependent on source habitat.

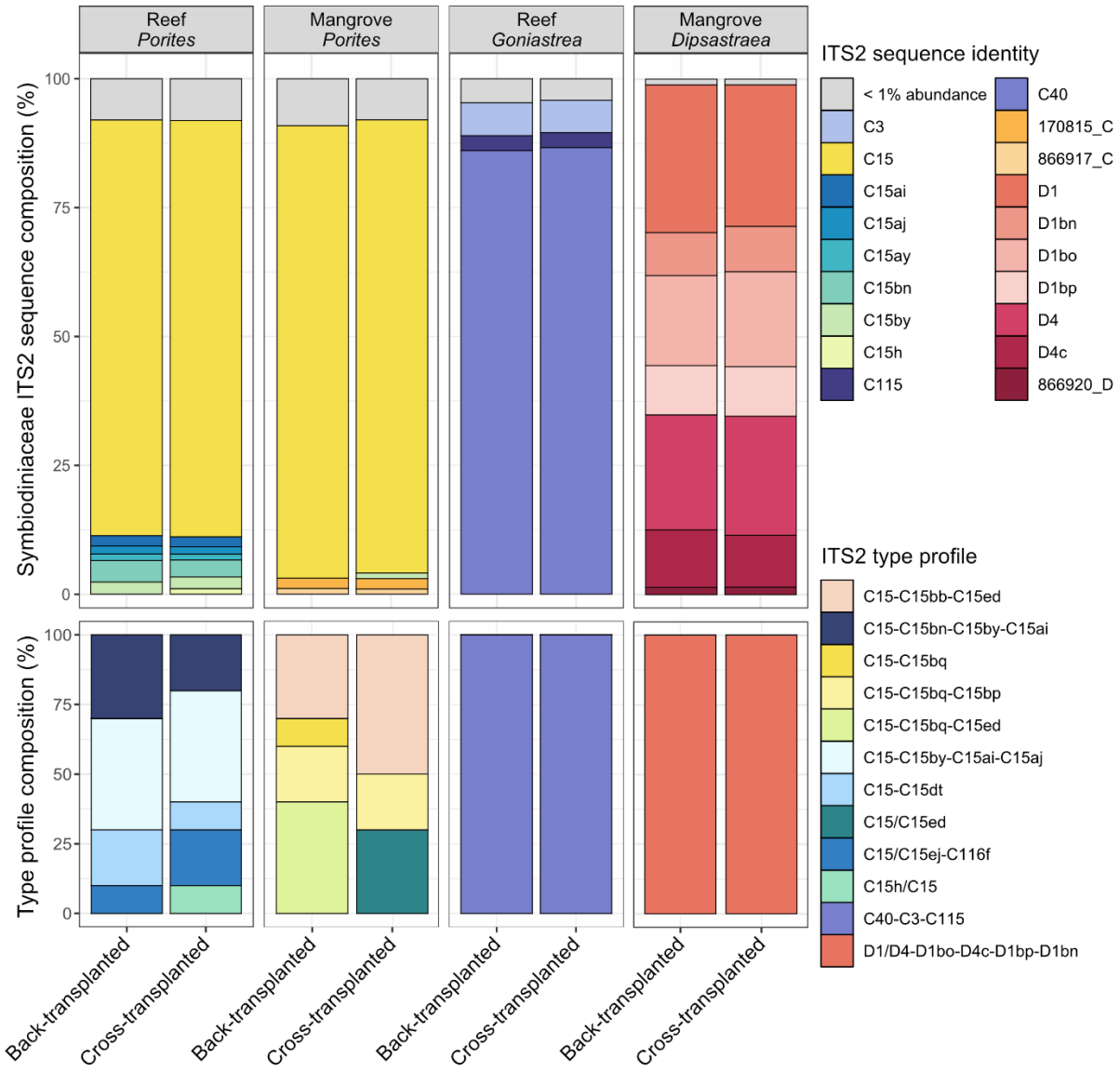


Figure 4.10. Average relative abundance (%) of Symbiodiniaceae ITS2 sequence variants (top panels) and ITS2 type profiles (bottom panels) for reef *Porites*, mangrove *Porites*, reef *Goniastrea*, and mangrove *Dipsastraea*, sampled 96 hours post-translocation. Sequences commonly found in the literature or assigned a name through the SymPortal framework have their assigned names (e.g. C3, C15, or C15ai). Unclassified sequences are assigned a unique ID from the SymPortal database with the corresponding Symbiodiniaceae clade (e.g. 170815_C refers to a sequence with the unique ID 170815 from clade C, or *Cladocopium* genus. Only sequences comprising greater than 1% average abundance have been assigned colours, rare sequences have been grouped as '< 1% abundance' and coloured grey. ITS2 type profile names are informative: capitalised letters denote the algal clade or genus of that putative taxon and hyphens separate the component defining intragenomic ITS2 sequence variants (DIVs) making up that profile, in decreasing order of abundance e.g. profile C15-C15bq refers to a genotype of clade C (*Cladocopium* genus), where the C15 sequence variant is most abundant, and C15bq sequence is next abundant. Symbiodiniaceae taxa characterised by co-majority abundances of component DIVs are denoted by a forward slash, e.g. C15/C15ed (Hume *et al.*, 2019).

4.3.7. Coral survival

The corals were revisited and surveyed for survival one year after antibiotic treatment and translocation. Translocation to a new habitat was found to significantly decrease the probability of survival of any coral (Binomial GLM: $\beta_{translocation}$ = -2.83, SE = 0.81, z(80) = -3.49, $P < 0.001$; Fig. 4.11). Whereas treatment with antibiotics had little effect on survivability after one year (Binomial GLM: $\beta_{antibiotic}$ = -0.90, SE = 0.68, z(80) = -1.31, $P > 0.05$; Table 4.2). Hence, there was no interactive effect of antibiotic treatment and translocation on coral survivability (Binomial GLM: $\beta_{antibiotic:translocation}$ = 1.25, SE = 1.08, z(80) = 1.15, $P > 0.05$).

It was found that 30% of *Porites lutea* survived translocation from reef to mangrove, as did 40% of control mangrove to mangrove *P. lutea*. Conversely, there was only 20% survival of *P. lutea* from mangrove to reef, compared with 90% survivorship of reef-native *P. lutea* (Fig. 4.11 A). Similar was found for the merulinids; only 10% of the mangrove-origin *Dipsastraea* sp. survived cross-transplantation to the reef, whereas 80% of the back-transplanted mangrove to mangrove corals survived (Fig. 4.11 B). Half of the *Goniastrea* fragments back-transplanted to the reef were alive upon revisiting one year later, while only 10% of cross-transplants to the mangrove had survived (Fig. 4.11 C).

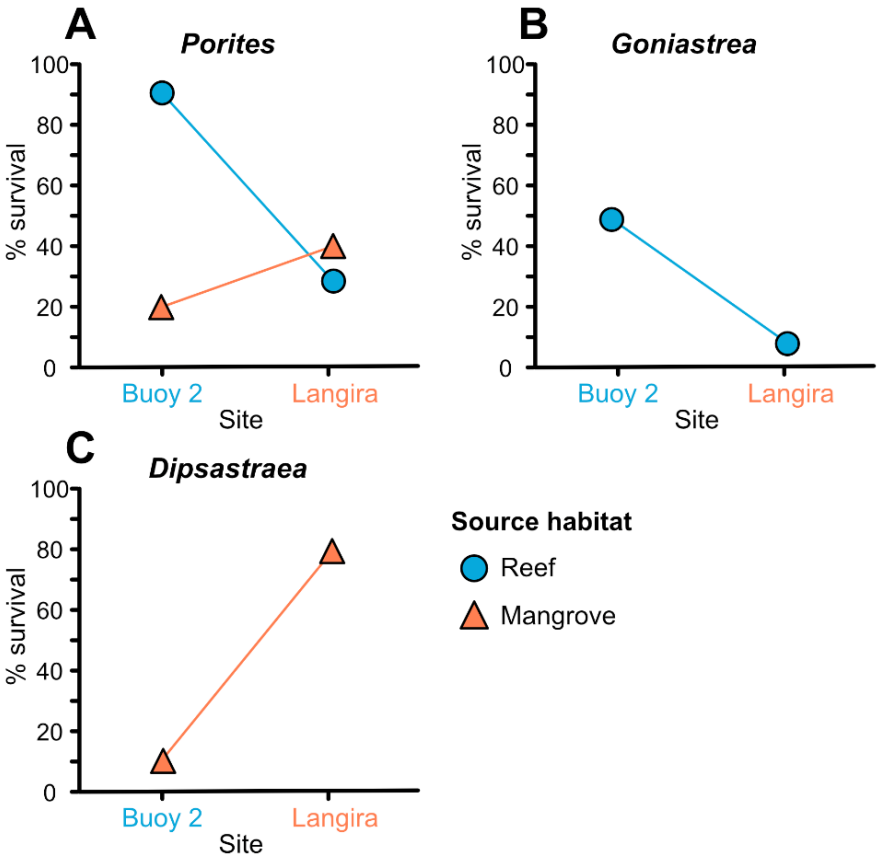


Figure 4.11. Interaction plot illustrating survival of coral transplants in the Wakatobi Marine National Park, Indonesia, one year after translocation. Symbol colour and shape denote source habitat of corals, x-axis shows sampled (destination) site. Each point represents percentage survival out of ten coral colonies.

Table 4.2. Survival summary of corals one year after antibiotic treatment and translocation.

Species and translocation	Treated	Untreated
Reef-reef *Porites*	5	4
Reef-mangrove *Porites*	1	2
Mangrove-mangrove *Porites*	2	2
Mangrove-reef *Porites*	1	1
Reef-reef *Goniastrea*	1	4
Reef-mangrove *Goniastrea*	1	0
Mangrove-mangrove *Dipsastraea*	4	4
Mangrove-reef *Dipsastraea*	1	0

All values are number of live coral colonies out of five, one year after translocation.

4.3.8. Thermal performance

The surviving *Porites lutea* appeared to have altered its thermal performance to suit the mangrove habitat, one year after translocation (Fig. 4.12). The optimum temperature (T_{opt}) for productivity of *P. lutea* in its native reef habitat was 32.79°C, whereas *P. lutea* which had been translocated to the mangrove habitat for one year exhibited an increased productivity T_{opt} of 34.33°C.

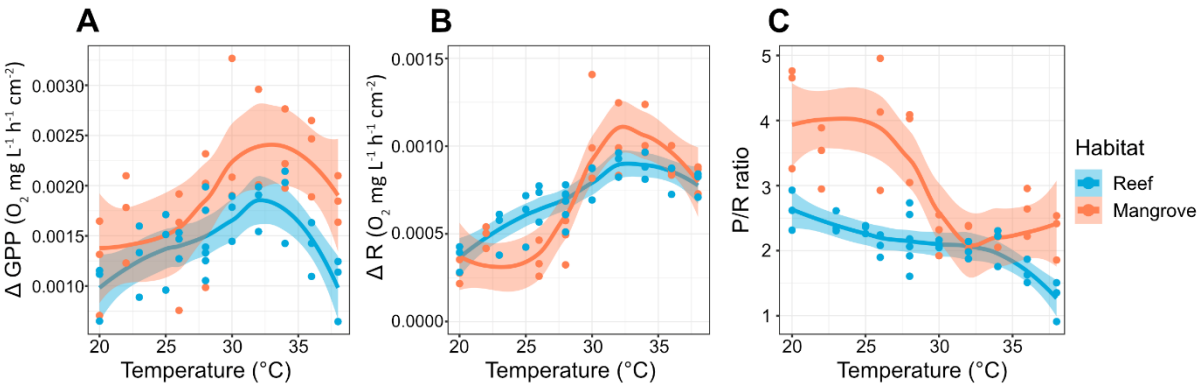


Figure 4.12. Thermal performance curves for reef-origin *Porites lutea* currently living in reef vs. mangrove habitat. Back-transplanted reef corals (*n* = 3) shown in blue, corals cross-transplanted to the mangrove (*n* = 3) shown in orange. Values are change in **A)** Gross primary productivity; **B)** Respiration; and **C)** Productivity:Respiration ratio, between ambient and assay temperature.

4.4. Discussion

4.4.1. Contrasting reef and mangrove habitats

The environmental conditions and benthic characteristics of Langira mangrove are very different to those of Buoy 2 fore-reef. While Buoy 2 hosted a species-rich assemblage of hard corals, dominated by branching *Porites* (consistent with a previous study; Caras & Pasternak, 2009), there were only five discernible coral species found in Langira mangrove (though cryptic species may exist). The lack of branching corals in the mangrove habitat could be due to the stressful environmental conditions, as branching corals are often considered to be more sensitive to environmentally-induced stress (Loya *et al.*, 2001;

McClanahan *et al.*, 2004). The multiple environmental stressors of corals found in mangroves have been branded the ‘deadly trio’ and include high fluctuating temperatures, low fluctuating pH, and low dissolved oxygen concentrations (Camp *et al.*, 2017). Extreme temperatures recorded in the mangrove during this study ranged from a minimum of 24.64°C to a maximum recorded temperature of 37.71°C, with a maximum daily range exceeding 7°C (Fig. 4.2). This was in contrast to the more stable temperature conditions recorded at neighbouring Buoy 2 reef (minimum: 25.61°C, maximum: 31.37°C, maximum daily range: 3°C; Fig. 4.2). Nutrient concentrations in the mangroves, in terms of total dissolved carbon and nitrogen, were also consistently higher in the mangroves than on the reef (Fig. 4.3). Although it is unclear whether higher nutrient concentrations constitute an added stressor or a mitigating factor in allowing coral to proliferate in such an extreme habitat.

4.4.2. Identification of mangrove corals

Dipsastraea cf. *pallida* had a distinct phenotype in the mangrove with a pale green colouration, tentacles extended, and shared corallite walls, which is usually a distinguishing feature of other merulinids such as *Favites* and *Goniastrea* spp. (Veron, 2000; Fig. S4.2). Genetic identification of stony corals is made more challenging by the fact that there is not enough variation in the genetic marker commonly used for other animals, mitochondrial Cytochrome c oxidase I gene (COI; Forsman, 2003). Through Sanger sequencing the nuclear ribosomal partial 18S-ITS1-5.8S-ITS2-partial 28S region, the mangrove merulinid was revealed to belong to the XVII-B clade, which includes the genera *Dipsastraea* (formerly *Favia*) and *Coelastrea* (formerly *Goniastrea*, Fig. S4.1; Huang *et al.*, 2011, 2014). Closest relatives for which ITS rRNA sequences had been deposited in GenBank included *Coelastrea aspera* (98% sequence similarity, accession: MK332020, unpublished) and *Dipsastraea pallida* (96%, HQ203337; Huang *et al.*, 2011). The genetic identification of this coral highlights the importance of correctly identifying study species. The Scleractinia are a diverse Order which harbour many cryptic species (Huang *et al.*, 2011; Ladner & Palumbi,

2012; Warner *et al.*, 2015; Sheets *et al.*, 2018), so it is likely that many environmental phenotypes have been misidentified. It is especially important to accurately identify study species when attempting to test for the effects of habitat on presumed conspecifics. Any differences in response to the environment, or ability to survive in extremes, could in fact be due to genetic divergence between coral hosts, or genetically distinct coral populations (Barshis *et al.*, 2010; Kenkel *et al.*, 2013a). Coral taxonomy is constantly being revised (Huang *et al.*, 2011, 2014; Veron, 2013), and genetic markers of suitable resolution (species down to population-level) are needed to confirm morphological identifications.

4.4.3. Coral-associated bacterial communities are habitat-influenced but host-regulated

Coral-associated bacterial communities were found to be significantly habitat-influenced before coral translocation (Fig. 4.7; Fig. S4.4 B; Table S4.1). There was a natural difference in bacterial OTU richness and diversity hosted by corals of the same species (*Porites lutea*) living in reef versus mangrove habitat, before treatment or translocation (Fig. 4.6). *Porites lutea* from Buoy 2 hosted a bacterial community dominated by Oceanospirillales, in particular Hahellaceae, which includes the known endosymbiont, *Endozoicomonas* (Neave *et al.*, 2017a). Conversely, *Porites lutea* from Langira mangrove hosted a more OTU-rich, even bacterial assemblage, dominated by Rhodobacterales, Rhodospirillales, and green and purple sulphur bacteria (Fig. 4.6 & Fig. 4.8). Dominance of the coral microbiome by *Endozoicomonas* is often linked to healthy corals (Bayer *et al.*, 2013a; Bourne *et al.*, 2016), whereas members of the Rhodobacterales have been linked to coral diseases (Mouchka *et al.*, 2010; Gignoux-Wolfsohn & Vollmer, 2015; Ng *et al.*, 2015). However, there were no visual signs of disease on any of the corals sampled here, and care should be taken in many cases when inferring traits from 16S rRNA gene meta-barcoding. Green and purple sulphur bacteria are known to coexist in sulphide-rich anaerobic aquatic environments such as those found in mangroves, where they reduce carbon dioxide to carbohydrates through

photosynthesis, using hydrogen sulphide (or other inorganic sulphur compounds) as an electron donor instead of water (Van Gemerden & Mas, 1995). The green and purple sulphur bacteria may live in syntrophy with sulphate-reducing bacteria (that produce sulphides) such as Desulfovibrionales and Desulfobacterales, which were also found to naturally associated with *Porites* in the mangrove (Fig. 4.8). Green sulphur bacteria were previously found to constitute the most abundant microorganism in the skeleton of the coral, *Isopora palifera*, where they were purported to play roles in primary production and nitrogen fixation (Yang *et al.*, 2016, 2019).

Corals of different species living in the same habitat hosted different bacterial assemblages, highlighting a degree of coral host-microbiome specificity (Fig. 4.7, Fig. 4.8, Fig. S4.4, Table S4.1). Coral-associated bacteria are known to demonstrate phylosymbiosis and cophylogeny with their coral hosts (Pollock *et al.*, 2018). Phylosymbiosis can be defined as 'microbial community relationships that recapitulate the phylogeny of their host' (Lim & Bordenstein, 2020), while cophylogeny describes the congruence in evolutionary development of two or more organisms which have shared a long-term interaction e.g. host and symbiont (demonstrated by their phylogenetic trees; Avino *et al.*, 2019). Coral host species was found to be the single most important variable in structuring the coral microbiome, across coral mucus, tissue, and skeleton samples of 236 coral colonies from 32 scleractinian and 4 other cnidarian taxa (Pollock *et al.*, 2018). The microbiome of the coral, *Acropora tenuis*, was found to be highly host-genotype specific and maintained compositional stability irrespective of reduced salinity, elevated temperature, and elevated partial pressure of CO_2, thereby highlighting the need to control for coral host genotype when researching coral microbiomes (Glasl *et al.*, 2019). Host-genotype effects were controlled for in the current study by fragmentation of colonies before assignment to different treatments and translocations.

4.4.3.1. A novel bacterial coral symbiont

A potentially unique coral-bacteria symbiosis was uncovered after amplicon sequencing the coral-associated bacterial community of *Dipsastraea* cf. *pallida*. The unclassified spirochaete, OTU1, was found to associate in extremely high abundance with *Dipsastraea* cf. *pallida*, accounting for over 47% of the bacterial community in native corals. The association appeared to be host-specific rather than habitat-driven as the spirochaete was not present in *Porites lutea* from the mangrove, and still comprised almost 20% of the bacterial community in *Dipsastraea* cf. *pallida* after translocation to the reef. The closest relative of OTU1 found in the NCBI database (94.28% sequence similarity) was an uncultured bacterium from sediment of a pristine mangrove on the northeast coast of Brazil (accession: EU420442; Taketani *et al.*, 2010). Another close relative (93.86% identity; KY376315) was sequenced from the coral *Acropora hyacinthus* translocated into a thermally variable back-reef pool (Ziegler *et al.*, 2017). The closest related cultured type specimens were both anaerobic marine *Spirochaeta* species (88.22% NR_117137 and 88.14% NR_104732). The *Spirochaeta* genus contains saccharolytic bacteria capable of breaking down polycarbohydrates in aquatic environments by anaerobic fermentation (Leschine *et al.*, 2006). However, while non-pathogenic, *Spirochaeta* are known to be free-living, and the relatively low sequence similarity of OTU1 to other *Spirochaeta* species suggests that this putative coral symbiont belongs in an as yet unclassified genus (94.5% similarity threshold) or even family (86.5% similarity threshold; Yarza *et al.*, 2014). Spirochaetes have previously been found to dominate the bacterial community of the red octocoral, *Corallium rubrum*, though their functional contribution remains unknown (van de Water *et al.*, 2016). There are suggestions that, like the termite-spirochaete symbiosis, spirochaetes may play a role in carbon or nitrogen fixation in the coral holobiont (Lilburn *et al.*, 2001; Brune, 2014; Tokuda *et al.*, 2018; van de Water *et al.*, 2018b). Several other octocoral species from both shallow and deep, tropical and temperate waters have been found to associate with spirochaetes (Holm

& Heidelberg, 2016; Lawler *et al.*, 2016; Wessels *et al.*, 2017), but spirochaetes have previously only been found to associate in relatively low abundances with scleractinian corals (Frias-lopez *et al.*, 2002; Ainsworth *et al.*, 2015). A novel spirochaete has recently been found in association with the coral predator, the crown-of-thorns starfish (*Acanthaster planci*; COTS); the spirochaete was found to form a biofilm-like structure in the subcuticular space, between the cuticle and epidermis (Wada *et al.*, 2020). Its ubiquity across allopatric species of COTS implies that the symbiotic relationship arose around two million years ago, coupled with genome reconstruction data which supports the spirochaete's evolution as an extracellular symbiont of subcuticular spaces. While the contribution of the spirochaete, OTU1, to the *Dipsastraea* cf. *pallida* holobiont remains unknown, its high abundance and host-fidelity suggest it plays an important role, potentially in making carbon or nitrogen sources bioavailable to the coral host.

The habitat-specificity of the coral-associated bacterial community highlights its potential to influence the adaptive capacity of the coral holobiont, though stable associations are likely regulated by the coral host.

4.4.4. Coral-associated bacterial communities exhibit flexibility

The coral-associated bacterial communities studied here appeared to be environmentally regulated as they changed rapidly (within 96 hours) following coral translocation (Fig. 4.7). However, while the coral-associated bacterial communities reorganised rapidly to cluster based on the sampled (destination) habitats of the corals within four days of translocation (Fig. S4.6), they did not resemble the bacterial assemblages hosted by native corals at the start of the experiment (before treatment and translocation; Fig. 4.7). Rather, the coral-associated bacterial communities of all corals seemed to shift to a more disturbed assemblage with an increased relative abundance of opportunistic bacteria, including members of the Flavobacteriales, Rhodobacteriales, and Alteromonadales (Fig. 4.8).

Translocation has previously been found to disrupt the coral microbiome, leading to an increase in potential pathogens (Casey *et al.*, 2015; Roitman *et al.*, 2020). Moreover, reciprocal translocations of the Caribbean coral *Orbicella faveolata* to and from turbid reef environments similarly did not result in bacterial communities bearing resemblance to pre-transplanted coral microbiomes, even after six months (Roitman *et al.*, 2020). This highlights the importance of sampling before and after translocation, as opposed to only end-point analysis. While site-specific differences in the microbiomes of *Acropora digitifera* and *Acropora hemprichii* were observed following translocation, the bacterial community composition prior to translocation was not captured (Ziegler *et al.*, 2017, 2019). Pre-transplants in these studies may have had different bacterial assemblages to 17-month or 21-month transplants given that the coral microbiome is known to change with colony age and over time (Williams *et al.*, 2015; Sweet *et al.*, 2017b). The findings of the current study illustrate the considerable flexibility of the *Porites lutea*, *Goniastrea edwardsi*, and *Dipsastraea* cf. *pallida* microbiomes, compared with other previously studied coral species such as *Pocillopora verrucosa*, which lies at the other end of the coral microbiome flexibility scale (Pogoreutz *et al.*, 2018; Ziegler *et al.*, 2019).

While the native microbiomes of *Porites lutea* from mangrove and reef habitats have been characterised and compared between sites in New Caledonia (Camp *et al.*, 2020) and the Great Barrier Reef (Camp *et al.*, 2019), this is the first time that habitat-dependent reassembly of the microbiome, inferred from translocation, has been demonstrated in a mangrove setting. Bacterial taxa whose abundances were statistically predicted by mangrove habitat included *Arcobacter* and *Marinifilum* which comprised a larger percentage of the coral microbiome in native mangrove corals, and in those corals translocated to Langira mangrove, than Buoy 2 reef. These bacterial genera have both been found to significantly contribute to differences in the bacterial community structure of mangrove soils with differing dominant mangrove tree species (Marcos *et al.*, 2018). All described species of *Marinifilum* have originally been isolated from seawater or coastal sediments and are

characterized as being halophilic, facultatively anaerobic, and chemoorganotrophic, meaning they oxidise organic matter for energy – all ideal traits for existence in a mangrove (Na *et al.*, 2009; Ruvira *et al.*, 2013; Xu *et al.*, 2016; Fu *et al.*, 2018).

4.4.5. Coral-associated bacterial communities are highly susceptible to disturbance

It was hypothesised that if coral-associated bacterial community structure was dependent on the environment, then reduction of the native bacterial load should have resulted in re-colonisation from the local environment. While samples taken four days after treatment and translocation did show clustering of coral-associated bacterial communities by sampled (destination) habitat, which is suggestive of horizontal transmission of bacteria from the local environment (Fig. S4.6), there is little evidence to suggest that antibiotic treatment accelerated this. Bacterial loading of antibiotic treated corals, determined by qPCR, stayed relatively stable compared with the bacterial abundances associated with corals incubated without antibiotics (Fig. 4.5). The bacterial abundances of corals incubated without antibiotics increased significantly immediately after incubation, before falling to original levels if back-transplanted, or remaining high if cross-transplanted (Fig. 4.5). Orders of magnitude lower bacterial abundance in antibiotic treated corals (compared with no-antibiotic incubated corals) coincided with comparatively lower bacterial diversity (except mangrove *Porites* whose bacterial community diversity decreased following incubation *without* antibiotics; Fig. 4.6). By 96 hours post-treatment, bacterial loading had generally recovered to previous levels, but interestingly, following re-introduction to the environment, antibiotic treated corals hosted higher bacterial diversity in terms of OTU richness, and evenness, than corals incubated without antibiotics. This elevated bacterial diversity could reflect rapid uptake of bacteria from the environment, though it conferred no advantage in terms of coral survivability (Table 4.2).

In addition to the increase in bacterial loading following 36-hour incubation without antibiotics, the coral-associated bacterial community compositions changed drastically, illustrating their susceptibility to disturbance (Fig. 4.7 & Fig. 4.8). Members of Vibrionales increased in relative abundance dramatically from < 1.5% in any coral species studied to > 17% in *Goniastrea*, > 21% in *Dipsastraea*, and > 26% in *Porites* immediately after incubation without antibiotics (Fig. 4.8). A previous study found that inoculation of the Caribbean coral *Montastraea cavernosa* with *Vibrio coralliilyticus* resulted in not only a 35% increase in the relative abundance of other *Vibrio* species, but a secondary effect of increased bacterial richness, and increases in other opportunists such as *Rhodobacterales* and *Cytophagales* (Welsh *et al.*, 2017), similar to the subsequent disruption seen here (Fig. 4.8). It is possible that the experimental incubation of corals hindered their natural mucus sloughing as aged mucus sheets of *Porites astreoides* have been shown to exhibit high relative abundances of Vibrionaceae and Rhodobacteraceae (Glasl *et al.*, 2016). In addition to relative increases in bacterial opportunists, putative symbiont proportions were decreased by disturbance. The dominant bacterial taxa in the mangrove *Dipsastraea* microbiome, OTU1, which originally comprised almost half (47%) of the coral-associated bacterial community, only accounted for 5% of the total community after incubation without antibiotics, and 28% with antibiotics. Analogous to findings where stressors decreased the relative abundance of the bacterial symbiont *Endozoicomonas* (McDevitt-Irwin *et al.*, 2017), here, disturbance led to a decrease in the relative abundance of a putative bacterial coral symbiont.

Multivariate GLMs identified that there were some coral-associated antibiotic-resistant bacterial taxa. For example, *Erythrobacter,* which was present before treatment and is known to be resistant to nalidixic acid and streptomycin (Koblížek *et al.*, 2003), comprised a higher relative proportion of the coral-associated bacterial community of antibiotic treated corals than corals incubated without antibiotics (Fig. 4.9). Antibiotic resistant taxa were also found associated with the coral *Acropora muricata* following treatment with the antibiotic ciprofloxacin; the re-establishing bacterial community was dominated by bacteria which had

survived treatment and proliferated in the absence of natural bacterial competitors (Sweet *et al.*, 2011b). In contrast with observations from *Porites astreoides*, where depletion of bacteria by antibiotics caused bleaching and necrosis to corals returned to the reef (Glasl *et al.*, 2016), here, corals appeared to remain healthy after transplantation into the environment, even as the bacterial communities became more diverse (Fig. 4.6).

4.4.5.1. Study limitations

The 'pot effect' evident by comparing the bacterial loading of corals before treatment with those immediately after 36 h incubation without antibiotics was probably due to reduced waterflow preventing natural mucus sloughing (Fig. 4.5). Destabilisation of the natural mucus community of *Porites astreoides* has previously been recorded for corals kept in control aquaria without antibiotics (Glasl *et al.*, 2016). While the increase in bacterial loading of control corals incubated in seawater for 36h was unexpected, it was at least recorded, thereby highlighting the effect of experimental design on the outcome of the experiment. Most experiments only implement a before-after or control-treatment design. As such, unrecorded laboratory, tank, or batch effects might represent a significant source of error in experiments. It is therefore important to design experiments with this in mind, and to take measurements at specific time points to account for environmental/ acclimatisation effects which might impact the control group, in order to capture the full story. The Before-After-Control-Impact (BACI) design (Smith, 2002) has been widely implemented for environmental impact studies, and is regarded to be a statistically powerful design to disentangle true treatment effects from environmental noise (Smokorowski & Randall, 2017). If nothing else, the 'pot-effect' captured in this study illustrates the importance of water movement, regular flushing, and mucus shedding for maintaining coral microbial balance.

A limitation in the quantification method used for estimating bacterial loading was that qPCR methods can also amplify an unknown number of dead or non-replicating cells may have

also been counted. Nevertheless, orders of magnitude changes in bacterial abundance, and widespread reorganisation of the coral-associated bacterial community was recorded as a result of disturbance (incubation) and was somewhat ameliorated by broad-spectrum antibiotic treatment.

4.4.6. Coral-Symbiodiniaceae associations are host-specific

Coral-Symbiodiniaceae associations were more host-specific and stable than coral-bacteria associations. *Porites* and *Goniastrea* were found to faithfully associate with symbionts of the genus *Cladocopium*, while *Dipsastraea* from the mangrove consistently associated with *Durusdinium* symbionts (Fig. 4.10). Members of the genus *Durusdinium* (previously referred to as clade D *Symbiodinium*) are frequently cited for conferring heat tolerance to their coral hosts (Baker *et al.*, 2004; Rowan, 2004; Berkelmans & van Oppen, 2006), which makes sense given the thermal extremes corals must withstand in Langira mangrove (Fig. 4.2). However, other arguments have been presented for what dominance by *Durusdinium* symbionts may mean, including the possibility that they are 'ominous signs' of less-favourable environmental conditions or 'selfish opportunists' which take hold under stressful conditions (Stat & Gates, 2011). It has also been suggested that hosting *Durusdinium* boosts coral thermotolerance at the expense of reduced growth (Little *et al.*, 2004; Jones & Berkelmans, 2010), which could have repercussions for coral reefs facing simultaneous warming and sea-level rise. The association between *Porites lutea* and symbionts from the *Cladocopium* C15 lineage was typical of Indo-Pacific *Porites* species (Fig. 4.10; LaJeunesse, 2005). The specific association between *Porites lutea* and *Cladocopium* C15 lineage has been recorded in a number of studies (Chen *et al.*, 2019; Camp *et al.*, 2020; Tan *et al.*, 2020) and it has been suggested that *Cladocopium* C15 contributes to the thermal resistance of *Porites* spp. (LaJeunesse *et al.*, 2003; Fitt *et al.*, 2009; Fisher *et al.*, 2012). All colonies of *Goniastrea edwardsi* hosted one putative Symbiodiniaceae taxon (a *Cladocopium* with the ITS2 type profile C40-C3-C115). Little is known about this association,

though ITS2 records used to compile information about symbiont diversity on the Great Barrier Reef show *Goniastrea* associated with C40 and C3 sequence variants (Tonk *et al.*, 2013), consistent with this study.

Based on samples taken four days (T96) after translocation, it was assumed that coral-symbiont associations of *Goniastrea* and *Dipsastraea* remained stable as symbiont identities of back- and cross-transplanted fragments of the same colonies were the same (Fig. 4.10). This suggests that shuffling or switching of algal symbionts is not such an immediate response to environmental change as bacterial community reorganisation, if Symbiodiniaceae respond at all. These findings are in agreement with those from a latitudinal study of corals in the Red Sea, whereby algal symbionts were host-specific and conserved across latitudes, while the diversity and composition of the bacterial communities varied dramatically between sites (Osman *et al.*, 2020). Corals from the hottest reefs in the world, in the Persian/Arabian Gulf, were also found to exhibit symbiont fidelity, not flexibility, over 1.5 years, despite extreme seasonal warming and acute heat stress (≥ 35°C; Howells *et al.*, 2020). In the absence of any visual signs of environmental stress, it might be that the endosymbiotic algae themselves are highly physiologically plastic and able to survive a range of latitudes (Osman *et al.*, 2020), and environmental conditions.

The only habitat-attributable difference in Symbiodiniaceae recorded in this study was the difference in ITS2 type profile hosted by *Porites lutea* originating from reef versus mangrove habitat (Fig. 4.10). Similar such differences in the *Cladocopium* type profiles hosted by *Porites lutea* were recorded from closely located mangrove and reef habitats in New Caledonia (Camp *et al.*, 2020). Furthermore, *Porites lutea* originating from different habitats seemed never to share the same algal symbionts (based on ITS2 type profile), even when cross-transplanted. However, based on ITS2 sequence variants, and predicted type profiles, there were slight differences between the symbiont communities of back-transplanted and cross-transplanted *Porites*. The ITS2 sequence variant data suggests shuffling of rare

sequence variants could have contributed to these differences, while the predicted type profiles point toward evidence of symbiont switching (Fig. 4.10).

This raises questions about the accuracy of ITS2 type profile predictions as different conclusions can be drawn from comparing sequence variants or type profiles. SymPortal type profile predictions are based on the presumption that most corals only harbour one symbiont type (Goulet, 2006) and are based on co-occurrence of sequences within samples (Hume *et al.*, 2019). The more times certain ITS2 sequence variants are found together in the same sample, the more likely they are to have come from the same algal symbiont. Therefore, type profile predictions (putative taxon assignments) will become more accurate and reliable as the SymPortal database grows (i.e. the predictions are only as good as the data already in the database).

Mangrove *Dipsastraea* seemed to host an even assemblage of *Durusdinium* sequence variants, which would have been the conclusion made by previous ITS2 sequencing studies (hence the sub-clade lettering system). However, SymPortal type profile analysis concluded that due to the consistent occurrence of these sequences across different samples, the sequences probably belong to the same *Durusdinium* genotype (assigned the type profile: D1/D4-D1bo-D4c-D1bp-D1bn). This has implications for previous Symbiodiniaceae typing studies which might have come to different conclusions had more resolute sequencing and analysis been available. Since the reclassification of the Symbiodiniaceae into separate genera (LaJeunesse *et al.*, 2018), Symbiodiniaceae identifications can be corroborated by DNA sequencing other genetic regions such as the chloroplast large subunit (cp23S), or non-coding plastid psbA minicircle (psbAncr) (Goulet *et al.*, 2019).

The importance in resolving coral host-symbiont combinations lies with their ability to dictate the physiological response of corals to thermal stress (Hoadley *et al.*, 2019). Results from four coral species inhabiting offshore, and elevated temperature inshore habitats showed that all inshore corals hosted the thermally tolerant *Durusdinium trenchii* symbiont and had

relatively muted responses to heat stress compared with their *Cladocopium*-hosting offshore counterparts (Hoadley *et al.*, 2019). Congruent with findings by Camp *et al.* (2020), the potentially distinct genotypes (type profiles) identified for *Porites lutea* between habitats in the current study could be specifically adapted to the very different abiotic conditions experienced in Buoy 2 reef and Langira mangrove (Fig. 4.2 & Fig. 4.3). Taking into account the symbiont associations of all the species studied here, results suggest that different coral species have different strategies for surviving the environmental extremes presented by mangrove habitat, but that host-symbiont associations are more host-specific and temporally stable than host-bacteria associations.

4.4.7. Local adaptation of coral holobionts

The high mortality of corals cross-transplanted to a new environment compared with those back-transplanted within the same habitat suggests that corals were locally adapted to their native environments (Fig. 4.11). More colonies of *Porites lutea*, originating from either habitat, survived when back-transplanted into their local habitat, demonstrating a clear home-advantage. However, there was relatively high mortality (30-40% survivability) of *Porites lutea* at Langira mangrove irrespective of source habitat, indicating that Langira mangrove is a more stressful or lower quality habitat for *Porites* to live in. Similarly, mortality of the coral *Orbicella faveolata* was observed when translocated into more turbid habitat on Varadero Reef near Cartagena, Colombia, for six months (Roitman *et al.*, 2020). In the same study, corals transplanted to the marginalised turbid reef exhibited increased microbial diversity, and the authors suggested the corals were on the brink of dysbiosis (Roitman *et al.*, 2020).

On the other hand, *Dipsastraea* cf. *pallida* from the mangrove had much greater survivability (80% of colonies) when back-transplanted within the mangrove relative to those cross-transplanted to the reef (10% of colonies), suggesting that the mangrove-origin *Dipsastraea*

sp. is a mangrove habitat specialist. Whether this is linked to its unique association with the putative spirochaete (OTU1) remains to be answered. Since there was no significant effect of antibiotic treatment, nor interactive effect of antibiotic treatment and translocation on survivability of corals, it is difficult to say whether the microbial community had any influence on the degree of local adaptation exhibited by the corals studied here. Further studies to examine the local adaptation of corals living in marginal habitats should examine more proxies for coral fitness such as coral metabolism, photophysiology, calcification, protein content, growth rate or reproductive outputs. Following reciprocal translocations, these metrics could better determine any potential trade-offs for coral adaptation to marginal habitats.

4.4.7.1. Living in a mangrove habitat may acclimatise corals to warming seas

Results from a pilot study of three *Porites lutea* colonies translocated from Buoy 2 reef to Langira mangrove versus three colonies back-transplanted within the reef showed some promise of thermal acclimatisation to marginal coral habitat (Fig. 4.12). Coral colonies cross-transplanted from reef to mangrove habitat for a year exhibited higher thermal optima and thermal tolerance limits when subjected to acute thermal stress assays than coral colonies native to the reef. Thermal acclimatisation of *Acropora hyacinthus* was similarly observed following 12 to 27 month translocations, which was reflected in patterns of gene expression between genetically identical coral fragments transplanted to different thermal habitats (Palumbi *et al.*, 2014). The authors concluded that in less than two years, acclimatisation had achieved the same thermal tolerance which would be expected to occur over many generations of natural selection (Palumbi *et al.*, 2014). In a separate study of the same coral species and same study system (*Acropora hyacinthus* and the thermally variable back-reef pools of Ofu Island, American Samoa), the increased thermal tolerance of transplanted corals coincided with a shift in bacterial community composition of transplants to match the microbiota of corals native to the thermally extreme back-reef pools (Ziegler *et al.*, 2017).

The source of the elevated thermal performance recorded in the current study remains uncertain, though thermal acclimatisation of a long-lived coral, such as *Porites*, within one year provides some hope for the capacity of corals to withstand warming oceans.

4.5. Conclusion

In conclusion, this study highlighted the propensity of the coral microbiome to rapidly shift, not only dependent on habitat, but also with disturbance caused by treatment and transplantation (even within the same habitat). While the rapid reorganisation potential of the coral microbiome still holds some promise with regards to an intermediate adaptive process, and there may be microbes which provide useful traits or functions to corals living in extreme environments, there are very real risks involved in manipulation of the coral microbiome. Active interventions could represent significant disturbances to the coral microbiome, and therefore the health of the coral holobiont. This study illustrates that there is still much more to be learned about actively intervening in the coral microbiome. Scientists should proceed with caution and aim to gain a better understanding of the biology, but also calculate the risks involved, and consider the ethics of intervention, before implementation for conservation purposes.

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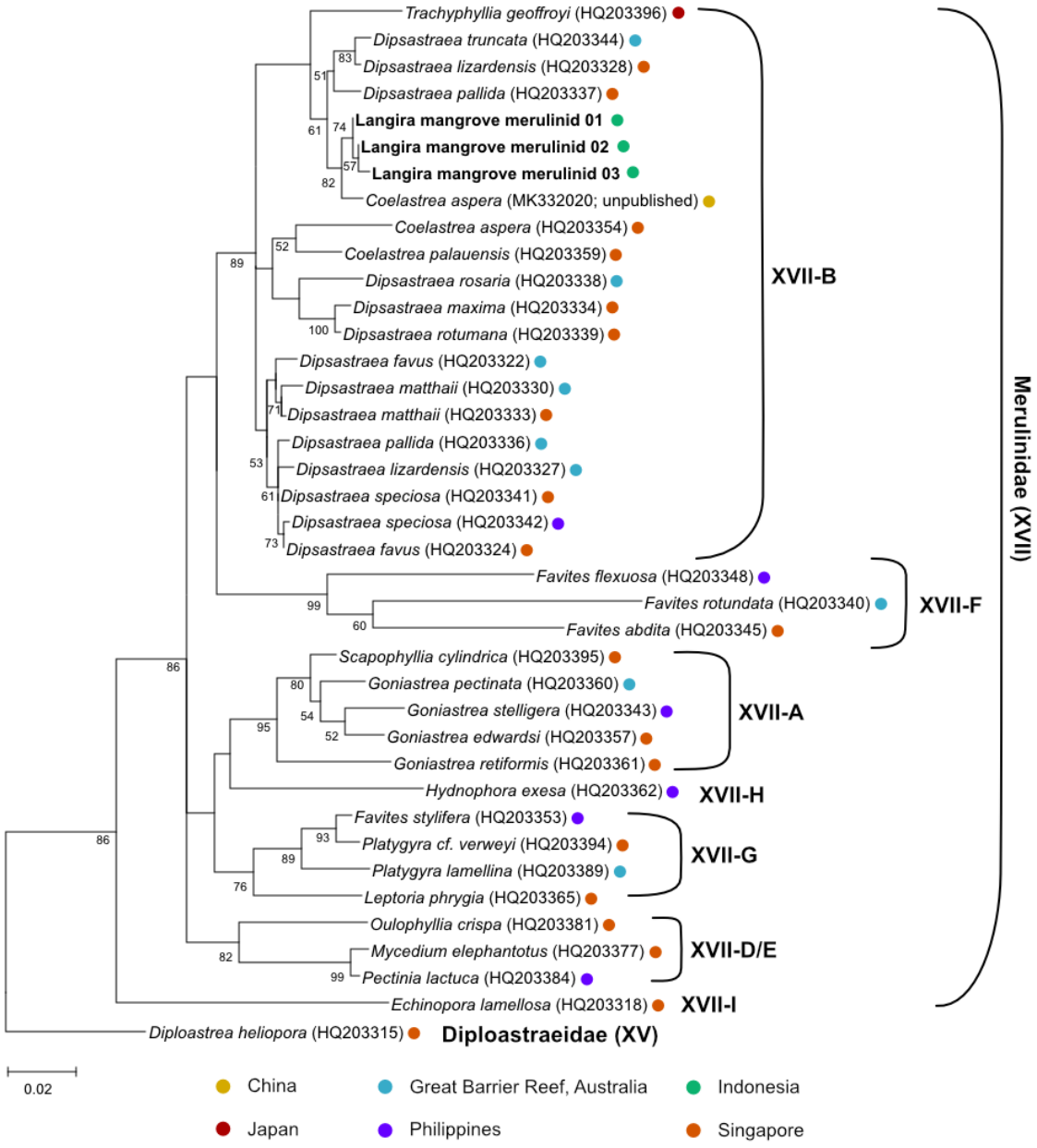
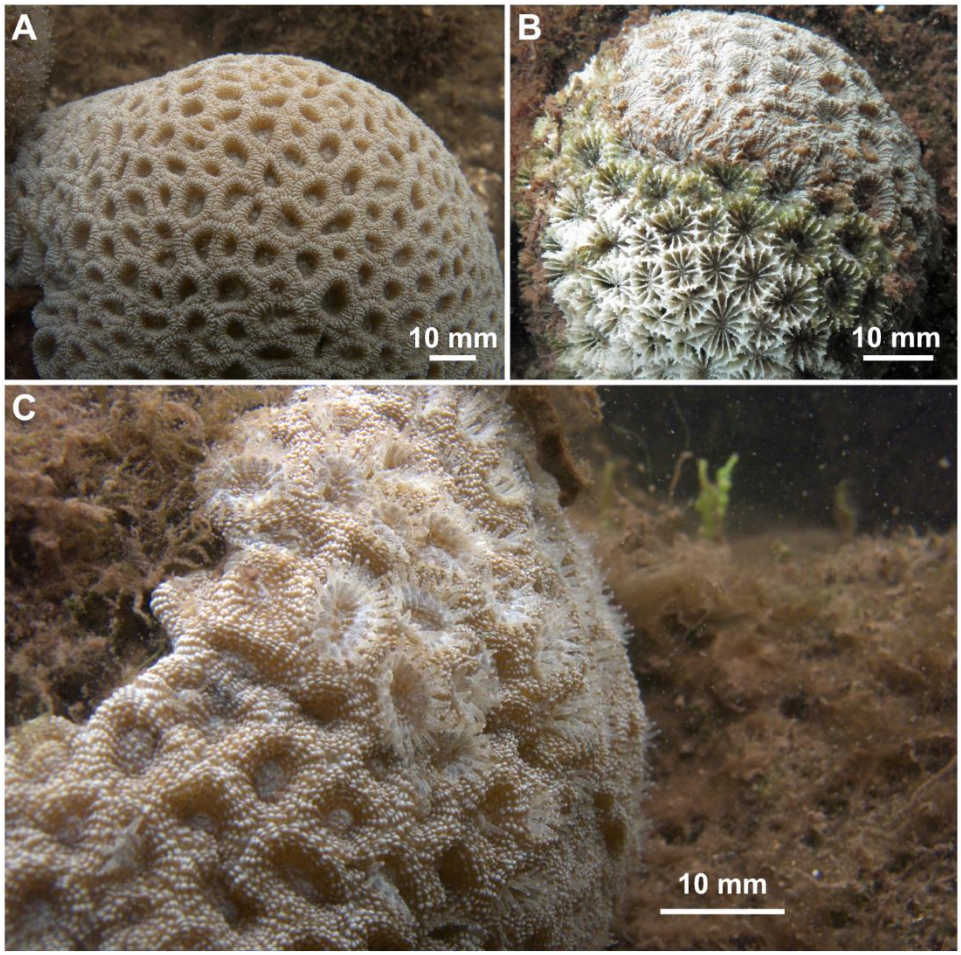
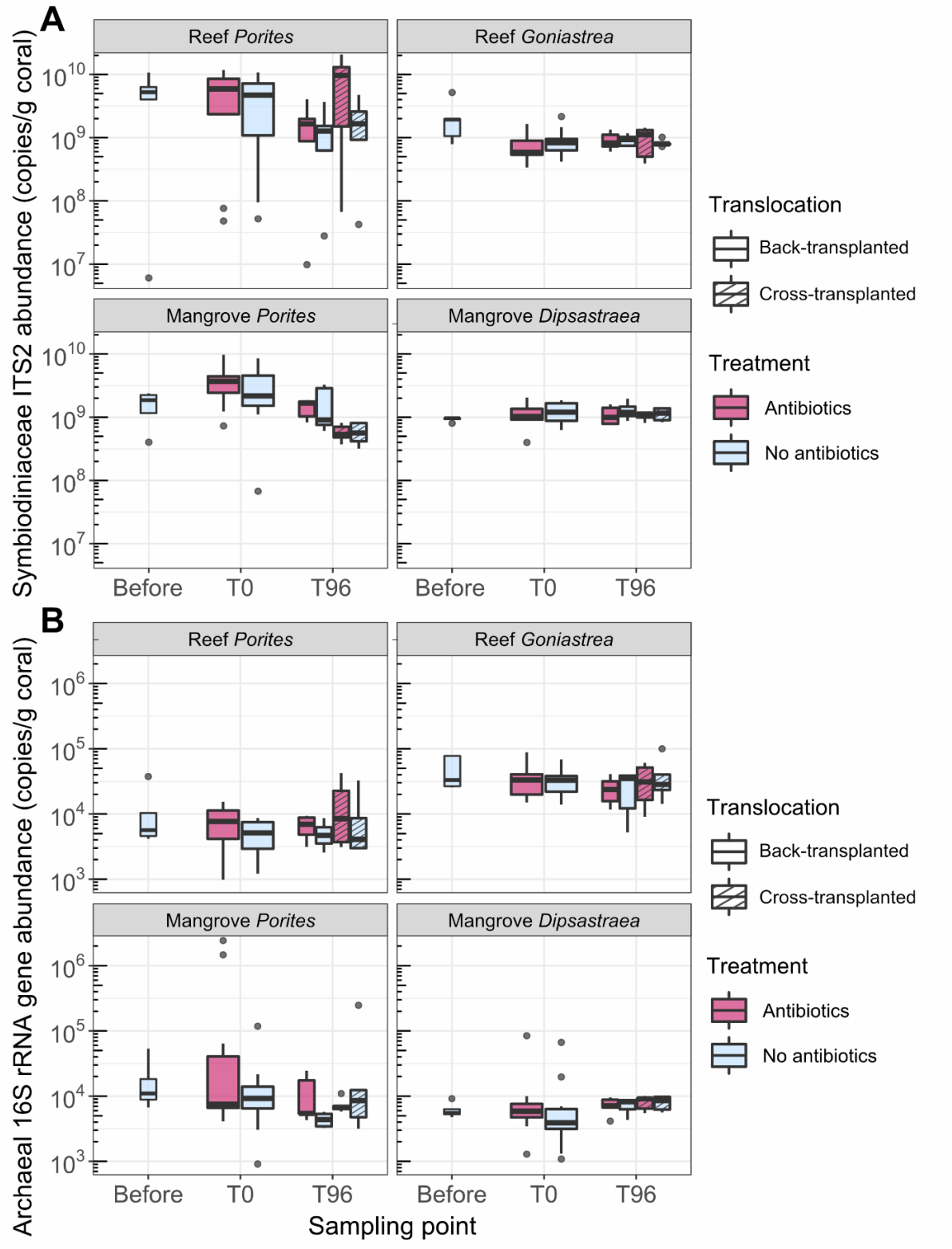
4.8. Supplementary material



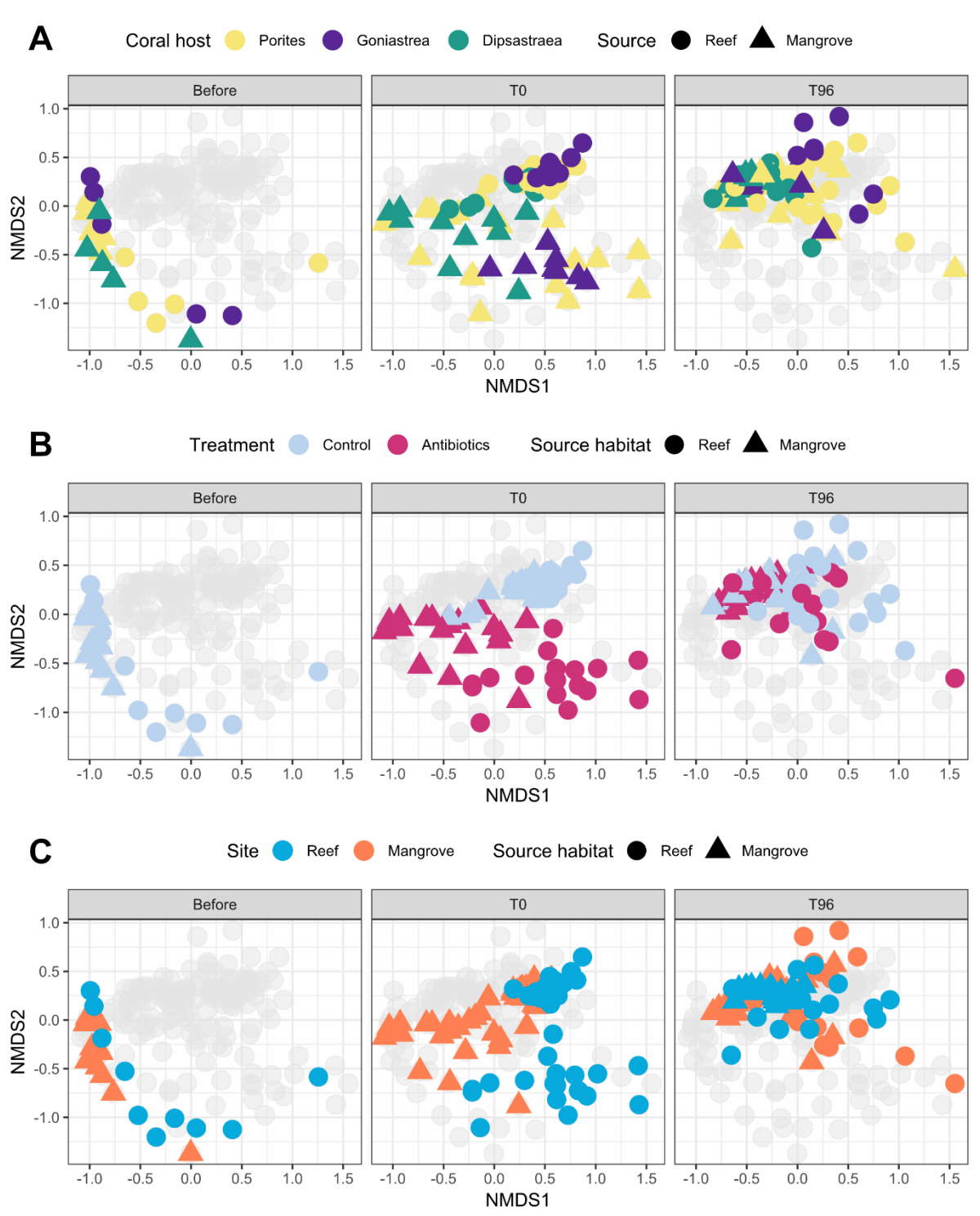
Supplementary figure 4.1. Phylogenetic tree showing mangrove *Dipsastraea* within the family Merulinidae, based on the internal transcribed spacer regions 1 and 2, including 5.8S rRNA gene. Evolutionary history inferred using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap replicates. Numbers adjacent to branches show maximum likelihood bootstrap support values (% of replicate trees in which the associated taxa clustered together; Felsenstein, 1985). Evolutionary analyses conducted in MEGA X (Kumar *et al.*, 2018). Outgroup was *Diploastrea heliopora* (family: Diploastraeidae) based on Huang *et al.* (2011 & 2014). Coloured dots show the country each coral sample came from. All reference sequences came from Huang *et al.* (2011), other than one unpublished *Coelastrea aspera* sequence from GenBank. Accession numbers shown in parentheses. Taxonomic rank and clade (roman numerals) shown to the right of the tree.



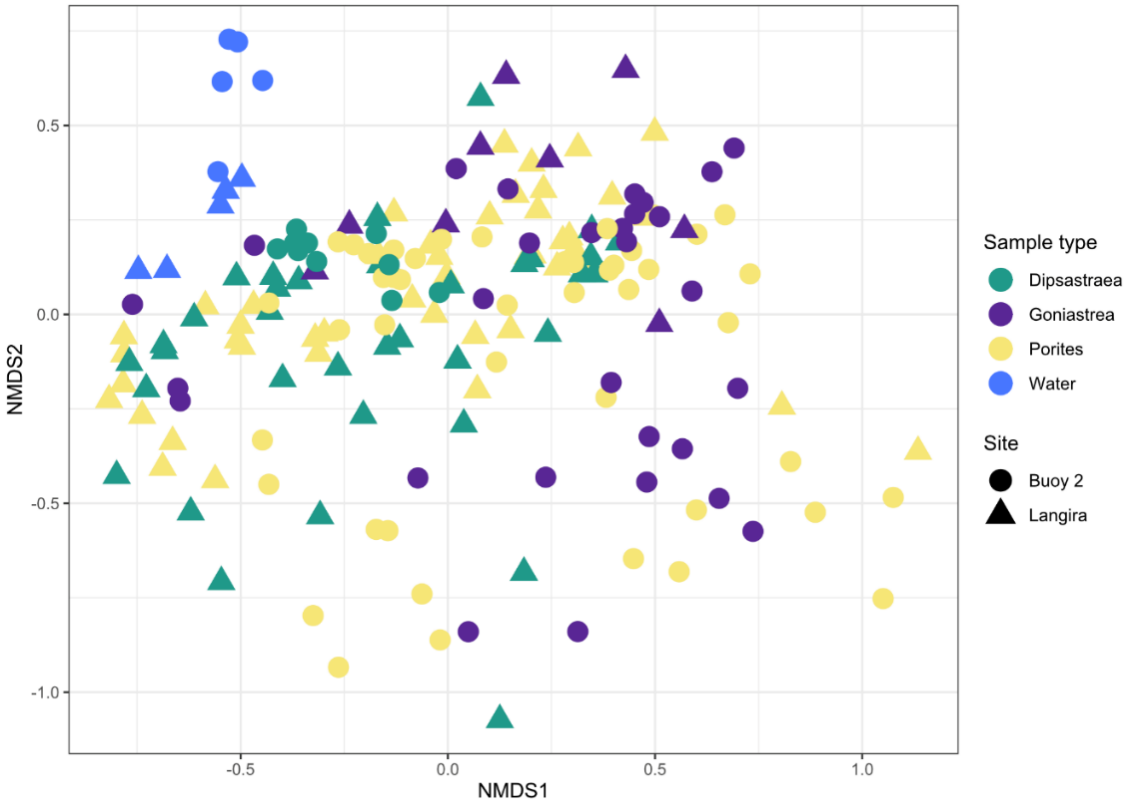
Supplementary figure 4.2. *Dipsastraea* cf. *pallida in situ* in Langira mangrove. **A)** Massive morphology with tissue giving the appearance of plocoid (distinct-walled) corallites. **B)** A diseased colony revealing the skeletal morphology including cerioid (shared-walled) corallites. **C)** Tentacles extended during the day.



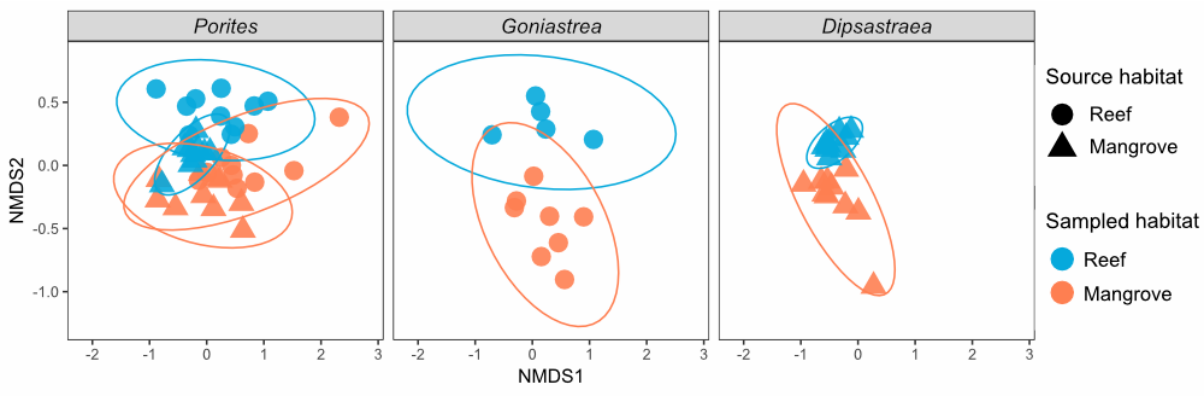
Supplementary figure 4.3. A) Symbiodiniaceae abundance (ITS2 region, ascertained by qPCR) **B)** Archaeal abundance (16S rRNA gene abundance) of *Porites lutea, Goniastrea edwardsi,* and *Dipsastraea pallida* before treatment, immediately after 36h incubation in antibiotics or seawater (T0), and 96 hours i.e. 4 days after treatment and translocation (T96). Antibiotic treated corals shown in pink, corals incubated in seawater-only shown in light blue, translocated corals with striped pattern. Boxplots depict median and interquartile range. Before treatment (native to habitat) represent *n* = 5 coral colonies, T0: *n* = 10 coral fragments, T96: *n* = 5 coral fragments.



Supplementary figure 4.4. Non-metric Multi-Dimensional Scaling (nMDS) ordination of bacterial community composition based on Bray-Curtis dissimilarity (2D stress = 0.19). Each point represents a sample. **A)** Points coloured by coral host species (yellow: *Porites lutea*, purple: *Goniastrea edwardsi*, green: *Dipsastraea* cf. *pallida*). **B)** Points coloured by treatment (light blue: seawater-only control, pink: antibiotic treatment). **C)** Points coloured by sampled habitat (blue: Buoy 2 fore-reef, orange: Langira mangrove). Shapes represent source habitat of coral hosts (circle: reef habitat, triangle: mangrove habitat). Sampling points separated by facets (Before treatment i.e. natural native community composition, T0: immediately after 36 h treatment with or without antibiotics, T96: Four days i.e. 96 hours after translocation).



Supplementary figure 4.5. Non-metric Multi-Dimensional Scaling (nMDS) ordination of bacterial community composition based on Bray-Curtis dissimilarity (2D stress = 0.19). Each symbol represents a sample, symbol colours denote sample type (blue: seawater, yellow: *Porites lutea* coral, purple: *Goniastrea edwardsi* coral, green: *Dipsastraea* cf. *pallida* coral), symbol shapes denote sampling site (circle: Buoy 2 fore-reef, triangle: Langira mangrove).



Supplementary figure 4.6. Non-metric Multi-Dimensional Scaling (nMDS) ordination of coral-associated bacterial community composition four days after translocation (T96) based on Bray-Curtis dissimilarity (2D stress = 0.16). Each symbol represents a sample, symbol colours denote sampled habitat (blue: Buoy 2 fore-reef, orange: Langira mangrove), symbol shapes denote source habitat (circle: reef, triangle: mangrove). Ellipses show 95% confidence intervals per grouping.

Supplementary table 4.1. Statistical comparison of the coral-associated bacterial community composition by permutational multivariate analysis of variance (PERMANOVA).

Before treatment PERMANOVA statistics			**Pairwise comparisons by habitat and coral host species.**				
Factor	**Model -F**	**P**		**Reef *Porites***	**Mangrove *Porites***	**Reef *Goniastrea***	**Mangrove *Dipsastraea***
Coral host species	3.411	**< 0.001**	Reef *Porites*	-	2.647	1.682	5.322
Source habitat	2.895	**< 0.001**	Mangrove *Porites*	**< 0.05**	-	1.936	5.143
			Reef *Goniastrea*	0.432	0.066	-	3.787
			Mangrove *Dipsastraea*	**< 0.05**	**< 0.05**	**< 0.05**	-

Immediately after treatment (T0) PERMANOVA statistics				
Factor	**Model-F**	**R^2**	**Df**	**P**
Coral host species	5.905	0.101	2	**< 0.001**
Source habitat	5.452	0.047	1	**< 0.001**
Antibiotic treatment	20.320	0.174	1	**< 0.001**
Coral species × Antibiotic treatment	3.136	0.054	2	**< 0.001**
Source habitat × Antibiotic treatment	2.921	0.025	1	**< 0.01**

Four days after translocation (T96) PERMANOVA statistics				
Factor	**Model-F**	**R^2**	**Df**	**P**
Coral host species	5.776	0.131	2	**< 0.001**
Source habitat	4.676	0.053	1	**< 0.001**
Antibiotic treatment	1.980	0.022	1	**< 0.01**
Sampled (destination) habitat	3.511	0.040	1	**< 0.001**
Coral species × Antibiotic treatment	1.043	0.024	2	0.366
Source habitat × Antibiotic treatment	0.707	0.008	1	0.901
Coral species × Sampled habitat	1.073	0.025	2	0.282
Source habitat × Sampled habitat	1.416	0.016	1	0.065
Antibiotic treatment × Sampled habitat	0.694	0.008	1	0.907
Coral species × Antibiotic × Sampled habitat	0.870	0.020	2	0.744
Source habitat × Antibiotic treatment × Sampled habitat	0.868	0.010	1	0.653

PERMANOVAs were conducted separately for each sampling point (Before, T0, and T96). Significant comparisons shown in bold. Pairwise comparisons between habitat and coral host species before treatment: upper values are model F-values, lower values are *p*-values adjusted for multiple comparisons.

Supplementary table 4.2. Bacterial genera found to be significantly differentially abundant between groups (one-way MV-GLM).

Bacterial genus (order)	One-way test statistic		
	Porites	***Goniastrea***	***Dipsastraea***
Aquihabitans (Acidimicrobiales)	66.41 *	50.76 ***	-
Ilumatobacter (Acidimicrobiales)	74.81 **	35.20 *	48.20 **
Alteromonas (Alteromonadales)	105.56 ***	50.76 ***	-
Ferrimonas (Alteromonadales)	66.81 *	35.67 *	48.20 ***
Idiomarina (Alteromonadales)	-	40.02 **	-
Marinobacterium (Alteromonadales)	93.75 **	35.20 **	48.20 **
Pseudoalteromonas (Alteromonadales)	108.32 ***	32.42 *	35.76 *
Pseudoteredinibacter (Alteromonadales)	-	42.87 **	-
Thalassotalea (Alteromonadales)	96.95 **	32.42 *	35.76 *
Carboxylicivirga (Bacteroidales)	-	32.73 *	-
Marinifilum (Bacteroidales)	61.99*	35.67 *	-
Pseudobacteriovorax (Bdellovibrionales)	68.61 *	32.42 *	35.76 *
Ralstonia (Burkholderiales)	81.10 **	32.42 *	35.76 *
Arcobacter (Campylobacterales)	84.14 **	50.76 ***	-
Oceanirhabdus (Clostridiales)	-	35.20 *	-
Vallitalea (Clostridiales)	-	36.38 *	-
Fabibacter (Cytophagales)	-	36.71 *	-
Desulfovibrio (Desulfovibrionales)	-	40.33 ***	-
Actibacter (Flavobacteriales)	67.85 *	-	-
Flavobacterium (Flavobacteriales)	66.15 *	35.20 *	48.20 **
Kordia (Flavobacteriales)	-	-	36.95 *
Mesoflavibacter (Flavobacteriales)	-	-	35.76 *
Polaribacter (Flavobacteriales)	-	-	37.92 *
Tenacibaculum (Flavobacteriales)	74.84 **	32.42 *	35.76 *
Pseudohaliea (Gammaproteobacteria_incertae_sedis)	-	-	36.94 *
Kordiimonas (Kordiimonadales)	68.31 *	35.20 *	48.20 **
Amphritea (Oceanospirillales)	68.03 *	50.76 **	-
Litoricola (Oceanospirillales)	83.20 **	35.20 *	48.20 **
Neptuniibacter (Oceanospirillales)	77.81 **	35.20 *	48.20 **
Oleibacter (Oceanospirillales)	-	30.86 *	-
Cohaesibacter (Rhizobiales)	73.62 *	35.67 *	48.20 **
Methyloceanibacter (Rhizobiales)	65.19 *	35.20 *	48.20 **
Donghicola (Rhodobacterales)	-	-	47.38 **
Marivita (Rhodobacterales)	71.48 *	35.20 *	48.20 **
Ruegeria (Rhodobacterales)	72.50 **	32.42 *	35.76 *
Shimia (Rhodobacterales)	103.47 ***	32.42 *	35.76 *
Tropicibacter (Rhodobacterales)	-	-	41.39 **
Pelagibacter (SAR11)	-	39.05 **	-
Aureispira (Sphingobacteriales)	-	-	51.76 ***
Lewinella (Sphingobacteriales)	-	-	48.20 **
Erythrobacter (Sphingomonadales)	75.81 **	35.20 *	48.20 *
Porphyrobacter (Sphingomonadales)	-	-	36.78 **
Methylophaga (Thiotrichales)	68.66 *	35.20 *	48.20 **

Allomonas (Vibrionales)	-	-	36.77 *
Photobacterium (Vibrionales)	81.69 **	35.20 *	48.20 **
Vibrio (Vibrionales)	-	-	45.20 **

Genera ordered alphabetically by taxonomic order and genus. Level of significance denoted by asterixis: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Most significant genera with highest relative abundances shown in bold and plotted in Fig. 4.9.

Chapter 5: Concluding remarks

5.1. Thermal biology of corals from marginal habitats

As coral reefs decline worldwide due to anthropogenically caused climate change and a raft of local stressors (Bellwood *et al.*, 2004; Veron *et al.*, 2009), there is an increasing urgency to find corals naturally able to survive extreme conditions. Research is turning toward corals living outside of typical pristine reef environments, in what are considered marginal habitats, which already present extreme conditions for coral survival (Camp *et al.*, 2018). Mangrove habitats with their 'deadly trio' of high fluctuating temperatures, low pH, and low dissolved oxygen represent natural laboratories or windows into the future of the impacts of climate change on stony corals (Camp *et al.*, 2017).

This thesis explored the thermal responses and microbial community compositions of coral holobionts living in contrasting reef and mangrove habitats in two different bioregions, the Western Indian Ocean, and the Central Indo-Pacific. The marginal mangrove sites located either side of the Indian Ocean hosted very different environmental conditions in comparison to neighbouring reefs. The temperatures recorded over the course of a year from both mangrove habitats in this thesis reached extreme highs and fluctuated daily (Table 5.1). The mangrove habitats studied here exhibited thermal regimes comparable to other highly thermally variable study systems such as the intertidal reef flats of the Kimberley Region, Western Australia (Schoepf *et al.*, 2015), and the back-reef pools of Ofu Island, American Samoa (Palumbi *et al.*, 2014; Barshis *et al.*, 2018; Thomas *et al.*, 2018), highlighting their utility as natural laboratories.

In the aftermath of the 2016 'Godzilla El Niño', surveys from contrasting reef and mangrove habitats within Curieuse Marine National Park, Seychelles showed some promising results (presented in Chapter 3:). While Home Reef was largely decimated by the marine heatwave,

Turtle Pond mangrove lacked dead coral and housed persistent, usually heat-sensitive, branching corals. Similar recovery from the 2016 mass-bleaching event was recorded for *Acropora aspera* living in macrotidal, thermally variable reef habitat in NW Australia (Schoepf *et al.*, 2020). This suggests that thermally variable coral habitats may provide refugia for corals facing more frequent and severe heating events (Oliver & Palumbi, 2011a; Schoepf *et al.*, 2020).

Table 5.1. Temperature summaries for reef and mangrove habitats in Curieuse Marine National Park, Seychelles, and the Wakatobi Marine National Park, Indonesia, from 2017 to 2018.

Bioregion	**Western Indian Ocean**		**Central Indo-Pacific**	
Site	**Home Reef**	**Turtle Pond mangrove**	**Buoy 2 fore-reef**	**Langira mangrove**
Absolute maximum temperature recorded (°C)	31.78	33.85	31.37	37.71
Absolute minimum temperature recorded (°C)	25.81	25.51	25.61	24.64
Largest daily temperature range (°C)	2.28 (27.37 - 29.65)	5.31 (26.88 - 32.19)	2.99 (27.57 - 30.56)	7.36 (30.36 - 37.71)

While corals living in mangrove habitats can naturally survive extreme temperature fluctuations *in situ*, their thermal tolerance limits had not yet been compared to conspecifics residing in typical reef habitats. The 20-day heat-ramping experiment presented in Chapter 2 was the first to experimentally test the thermal tolerance limits of the hardy reef-building coral, *Porites lutea*, from mangrove versus reef habitat. Somewhat surprisingly, when brought into a common-garden heating experiment, the corals from the mangrove did not perform significantly better under heat stress in terms of productivity, than the reef corals, although the mangrove corals did not bleach as severely as the reef corals. Since the mangrove corals survive regular extreme temperatures *in situ*, but did not fare much better than the reef corals under a common set of environmental conditions, it suggests that there could be something in the mangrove environment which mitigates the worst effects of warming. Several studies have hinted at the role of trophic plasticity and switching to a more

heterotrophic lifestyle as a strategy to survive environmental extremes whilst algal symbionts are compromised (Anthony & Fabricius, 2000; Grottoli *et al.*, 2006; Houlbrèque & Ferrier-Pagès, 2009; Morgan *et al.*, 2016; Camp *et al.*, 2020; Conti-Jerpe *et al.*, 2020). Anecdotal evidence of *Dipsastraea* cf. *pallida* from Langira mangrove with its tentacles perpetually extended supports this notion (Fig. S4.2; pers. obs.). The mechanisms by which upregulation of heterotrophy could save corals from the extreme conditions of mangroves, and extreme conditions expected on future reefs, warrants further work. Future work could involve defining trophic niches using stable isotope analysis (Conti-Jerpe *et al.*, 2020) and laboratory studies including feeding and heating assays with and without provision of food (Burmester *et al.*, 2018).

This thesis did provide some evidence for the acclimatisation potential of coral translocated to a more thermally variable mangrove habitat. *Porites lutea* originating from Buoy 2 fore-reef was found, through acute heating assays, to have increased its thermal performance optima following just one year living in the thermally extreme Langira mangrove, Indonesia (Chapter 4).

Going forward, there is a need to summarise the vast yet disparate literature covering the thermal biology of stony corals. This could be achieved through a comprehensive meta-analysis distilling the variety of response variables measured into response ratios or thermal performance curves (such as those piloted in Appendix 1). Information on the thermal niche of individual coral species and populations from different habitats would be a valuable resource if made open-access and available to reef managers (like the Coral Trait Database; Madin *et al.*, 2016). To make findings more easily comparable, researchers should develop a standardised method for measuring thermal performance, which is cheap and easy to replicate. The Coral Bleaching Automated Stress System (CBASS) or ‘coral in a box’ short-term acute heat stress assays, similar to those developed in Chapter 2 (Fig. 2.4) and Appendix 1, show some promise in this endeavour (Voolstra *et al.*, 2020).

It is important to note that coral identification is difficult and rarely clear-cut, even by genetic methods, so the crucial step in confirming the identity of a study species is often bypassed. If there is no accurate way of knowing which species was studied, it makes research almost impossible to replicate, as has already been discussed by entomologists (Owens, 2018). This could have already led to a catalogue of errors if traits have been attributed to the wrong species or to differences in holobiont composition (e.g. different symbiont clades or microbiota) when the host species or genotype may have contributed. One way to control for the potential effects of host genotype within studies, since corals are clonal organisms, is to fragment a colony for assignment to different experimental treatments, as was done in Chapter 4. The correct identification of coral species ensures that data available to other researchers who wish to replicate results and to reef managers involved in preserving species is accurate.

5.2. Coral-associated microbial communities are habitat-dependent

Scleractinian corals form meta-organisms with a multitude of associated microorganisms, which the coral hosts rely upon for energy (Muscatine, 1990) and nutrient provision (Bourne *et al.*, 2016), as well as defence against disease (Shnit-Orland & Kushmaro, 2009). The flexibility of the coral-microbiome relationship has led researchers to believe that microbes might be key in influencing the ecological success of corals in certain habitats and under certain environmental conditions (Reshef *et al.*, 2006; Voolstra & Ziegler, 2020). Therefore, this thesis sought to examine the relationships between coral hosts and their microbial symbionts in reef and mangrove habitats. The microbial constituents of the coral holobiont were explored with regard to the habitat the coral host originated from, and the environment sampled, before and after coral translocation. These translocation experiments were conducted in two biogeographic regions: the Seychelles, Western Indian Ocean (Chapter 3),

and Indonesia, Central Indo-Pacific Ocean (Chapter 4). Both studies revealed clear habitat-dependent differences in coral-associated bacterial communities. Notably, the bacterial community compositions of the same coral species, *Porites lutea*, living in reef and mangrove habitat, were naturally significantly different, and this was true of corals irrespective of biogeography (Fig. 3.9 & Fig. 4.7). Habitat-driven differences in the microbiomes of conspecific corals have previously been demonstrated for a number of coral species (e.g. *Acropora hyacinthus*: Ziegler *et al.*, 2017; *Acropora hemprichii:* Ziegler *et al.*, 2019; *Acropora muricata*, *Acropora pulchra*, and *Porites lutea*: Camp *et al.*, 2020), though this microbiome flexibility is not exhibited by all stony corals (e.g. *Pocillopora verrucosa*: Pogoreutz *et al.*, 2018; Ziegler *et al.*, 2019). This thesis also recorded some commonalities between the bacterial community compositions of corals living in mangrove habitats, across bioregions. Members of the Order Rhodobacterales comprised a larger proportion of the bacterial community in mangrove-dwelling corals, than their reef counterparts. Likewise, *Marinifilum* (Bacteroidales) and *Arcobacter* (Campylobacterales) occurred in higher relative abundance in corals from mangrove habitats in the Seychelles (Chapter 3) and also increased in relative abundance following translocation to mangrove habitat in Indonesia (Chapter 4). On the other hand, the bacterial Family Hahellaceae, which contains the known endosymbiont *Endozoicomonas*, comprised a comparatively smaller percentage of mangrove corals' microbiomes compared with corals from reef habitat (Fig. 3.10 & Fig. 4.8). What remains unclear, since many of the mangrove-associated bacteria have previously been linked to coral disease (Frias-Lopez *et al.*, 2002; Mouchka *et al.*, 2010), is whether the mangrove-influenced coral-associated bacterial communities benefit their coral hosts or hinder performance. Experimental manipulation of the coral microbiome involving selective removal (antibiotics) or inoculation (probiotics) of bacteria under a range of specific laboratory-controlled environmental conditions, as well as testing of Koch's postulates might go some way to disentangling the role of specific bacteria within the holobiont (Work & Meteyer, 2014).

5.2.1. A novel bacterial symbiont

An interesting finding of this project was the potential discovery of a novel bacterial coral symbiont – an unclassified Spirochaete associated with *Dipsastraea* cf. *pallida* from Langira mangrove in the Wakatobi Marine National Park, Indonesia (Chapter 4). Under natural conditions, before translocation, this particular OTU accounted for almost half of the coral's bacterial community. In order to further characterise this putative symbiont, a taxon-specific molecular probe could be developed for fluorescence *in situ* hybridisation (FISH) to visualise where, and in what tissues, within the coral host this bacterium resides. More in-depth sequencing (meta-genome for genome assembly or multi-locus sequence typing) could be conducted to produce an accurate phylogeny of this OTU within the poorly resolved Spirochaetes. Genome assembly and subsequent transcriptome or proteome analyses would also allow investigation of the active functional genes to gain more insight into its role within the coral holobiont.

5.2.2. Algal symbionts exhibit habitat-specificity and host-fidelity

Symbiodiniaceae, the algal coral endosymbionts, were found to exhibit both habitat-specificity and host-fidelity. *Porites lutea* consistently associated with algal symbionts of the genus *Cladocopium*, specifically from the C15 lineage, across habitats and bioregions. However, ITS2 type profile analysis revealed the potential presence of distinct *Cladocopium* genotypes hosted by corals from different habitats (Chapter 3 & 4); a phenomena also recorded for mangrove and reef-dwelling corals of the Great Barrier Reef (Camp *et al.*, 2019) and New Caledonia (Camp *et al.*, 2020). The merulinid corals from the Wakatobi Marine National Park, studied in Chapter 4, hosted different algal symbionts, with *Goniastrea edwardsi* from the reef hosting *Cladocopium*, and *Dipsastraea* cf. *pallida* from the mangrove hosting *Durusdinium.* Neither coral species swapped symbiont type after 4 days in a new habitat. In fact, the majority of corals studied, on either side of the Indian Ocean, showed

host-fidelity, at least in the short-term (and in the Seychelles one year), even after translocation (Fig. 3.13 & Fig. 4.10). This adds to a growing understanding that corals do not associate as flexibly with their algal symbionts as they do with their other microbial partners (Goulet, 2006; Stat *et al.*, 2009; Osman *et al.*, 2020). The different symbiotic strategies employed by corals living in marginal habitats requires further study to better understand the advantages and potential trade-offs of hosting particular symbionts. A key variable to measure would be the growth rates of corals living in marginal habitats. Research has suggested that hosting *Durusdinium* algal symbionts can increase thermotolerance, and thus the ability to survive warming oceans, at the expense of coral growth (Little *et al.*, 2004; Jones & Berkelmans, 2010). Marginal coral habitats, such as mangroves, may in this respect provide a window into a future where corals survive with the inability to form reefs or keep up with sea-level rise.

5.3. Rapid reorganisation of the coral holobiont

In comparison with the algal symbionts, the coral-associated bacterial community composition changed rapidly after exposure to a new environment. Within 44 (Chapter 3) to 96 hours (Chapter 4) after translocation, the coral-associated bacterial communities had reorganised to the extent that they were dissimilar to pre-translocated communities. Coral microbiome flexibility has been suggested to be key to allowing corals to rapidly respond to environmental change (Voolstra & Ziegler, 2020). With this is mind, antibiotic treatment was trialled as a means of accelerating the bacterial reorganisation process. An unintended consequence of attempting to control for handling effects by maintaining corals both with and without antibiotics in the same manner was that incubation without antibiotics had a stronger effect than treatment with antibiotics on the coral-associated bacterial community. This highlighted an important issue in that the coral microbiome is seemingly very sensitive to any disturbance, including aquaria conditions and handling (Kooperman *et al.*, 2007; Ainsworth & Hoegh-Guldberg, 2009; Glasl *et al.*, 2016). Therefore, any rapid reorganisation of the coral-

associated bacterial community may only signify the response of a disturbed microbiome. That is, the bacteria which are able to proliferate opportunistically take advantage of a change in environmental conditions, which could eventually lead to dysbiosis and disease or bleaching.

5.3.1. Can the coral microbiome confer adaptive advantages?

With regards to whether microorganisms could help corals adapt to environmental change (as discussed in Torda *et al.*, 2017; Fry *et al.*, 2020; Voolstra & Ziegler, 2020), it is still uncertain whether the coral-associated bacteria present in certain habitats or under certain environmental conditions are of any advantage to the coral, or whether the bacteria are responding opportunistically. In order to better understand whether microbiome restructuring can aid coral survival, researchers must go further than just revealing what microorganisms are present, and discover what the microorganisms are doing. Sequencing technology has made amplicon sequencing or 'meta-barcoding' (particularly of the 16S rRNA gene), accessible and affordable, so there is now a wealth of data available detailing the composition of various coral microbiomes under different conditions (Coral Microbiome Database: Huggett & Apprill, 2019; Global Coral Microbiome Project: Pollock *et al.*, 2018). However, moving beyond meta-barcoding (performed in this project) and meta-genomics (to see what genes are present), involves more functional approaches such as meta-transcriptomics – to see which genes are actively being expressed. It might be that the identity of the microorganisms does not matter so much as the roles they play (functional redundancy; Kimes *et al.*, 2010).

It is vital to determine where microbiome flexibility takes place within the coral, to help understand whether and how microorganisms might help corals adapt to rapid environmental change. It stands to reason that the surface mucus layer being the interface between coral and environment might be most significantly impacted by environmental change, but also the

least tightly linked with host functioning and therefore least likely to confer adaptive advantages to the coral host, due to its transient nature. This could be explored using fluorescence microscopy techniques, such as FISH. The bacterial coral endosymbiont, *Endozoicomonas* was found using catalysed reporter deposition–fluorescence *in situ* hybridisation (CARD–FISH) to form aggregations deep within its coral host's tissues, which coincides with its host-specificity (Neave *et al.*, 2017b), and cophylogeny (Pollock *et al.*, 2018). Furthermore, microorganisms found in coral tissue samples were found to be more strongly influenced by host traits than the microbiome of the coral mucus which was more influenced by environmental and ecological conditions (Pollock *et al.*, 2018). It is therefore important to compartmentalise coral microbiome samples into mucus, tissue, and skeleton (Sweet *et al.*, 2011a).

Further work to improve our understanding of the relative contribution of coral host versus microbial community under a range of environmental conditions might involve controlled laboratory studies, such as those implemented in mice and other host-microbiome model systems. For example, axenic culturing of corals or microbiome transplant studies (rather than mouse faecal transplant studies) could be used to disentangle complex causes and effects (Giraud, 2008; Lai *et al.*, 2018).

5.3.2. Local adaptation

Results of reciprocal translocations reported in this thesis supported the idea that corals are locally adapted to their environment. Cross-transplantation to a new habitat more frequently resulted in the ultimate trade-off of mortality, compared with corals which were back-transplanted within their local habitat (Fig. 4.11). While initial microbiome disturbance (by antibiotic treatment) was not a significant contributing factor to coral mortality in this study (Chapter 4), it is still not clear whether local adaptation is a result of the coral host genotype alone or a combination of host and microbial community. Future translocation studies to test

for local adaptation could also include further proxy measurements of fitness, including metabolism, photophysiology, calcification, growth, tissue protein content, or reproductive outputs. If corals are locally adapted to marginal habitats, then there is value in conserving these pockets of stress-resilient corals. Since typical reefs have begun to rapidly decline researchers have begun to realise the value that marginal coral habitats might hold (Rivest *et al.*, 2017). Turbid nearshore environments in the Coral Triangle have previously been suggested to provide refuge from climate change, but due to their close proximity to human populations, will need enhanced conservation status (Guest *et al.*, 2016; Sully & van Woesik, 2020). Even if the corals living in mangrove habitats are locally adapted and show little potential for acclimatisation to new environments, their unique mangrove habitats warrant conserving for their potential as climate refugia or reservoirs of climate-resilient corals.

5.4. Active interventions for coral conservation

As reef-building corals struggle to keep up with the pace of anthropogenically caused climate change, it has become more apparent that inaction is not an option. It was predicted that by 2050, 75% of the world's corals would be highly threatened (Burke *et al.*, 2011). A lot of research focus is now turning toward what active interventions we may be able to implement to slow the decline of coral reefs (Anthony *et al.*, 2017). While there are arguments by many scientists that this is obfuscating the problem of global warming and creating false optimism (Hughes *et al.*, 2017), it is surely better to at least explore the feasibility of all options before they are needed (Anthony *et al.*, 2017). Scientists have an obligation to provide governments and management bodies with the evidence to make sound decisions and implement legislation, but the underlying scientific basis first needs to exist.

The majority of active intervention research has centred around coral restoration involving coral gardening. Until now, most of that attention has been given to farming corals in pristine clear-water environments to grow corals as fast as possible. However, this may prove to be

a waste of resources, in terms of time, effort, and money, if the majority of farmed corals die in the next mass-bleaching event; which are increasing in frequency and are eventually expected to occur with every hot summer (Hughes *et al.*, 2018b). The cost of coral restoration on average has been estimated at $400,000 ha^{-1}, with most projects covering only small spatial scales (~100m^2) over short time scales (1-2 years), and mortality of restored corals averaging 40% (Bayraktarov *et al.*, 2019). Increasing the biomass of corals in already extreme environments, such as marginal coral habitats, could then represent a more sensible use of resources. While some research groups focus on selectively breeding corals to become more stress-resistant and/or resilient (van Oppen *et al.*, 2015, 2018), others may focus their efforts on preserving or building up a climate resilient stock of corals in marginal habitats (Morikawa & Palumbi, 2019). The research presented in this thesis highlights the potential conservation value of corals persisting under the extreme conditions of mangrove habitats. There is also the potential for the assisted evolution of coral symbionts due to their short generation times (van Oppen *et al.*, 2015; Chakravarti & van Oppen, 2018), or the inoculation of corals with more tolerant symbionts, providing associations remain stable (Mieog *et al.*, 2009b).

While this thesis demonstrates the potential for corals to horizontally uptake bacteria from their surroundings, another avenue of active intervention research involves the inoculation of corals with so-called Beneficial Microorganisms for Corals (BMCs; Peixoto *et al.*, 2017; Rosado *et al.*, 2018). How to choose the bacteria which make up a probiotic mixture is one of the key challenges in developing this idea. By studying the natural bacterial community compositions of corals living in challenging environments, such as those presented in this thesis, researchers may be able to learn more about which bacteria are truly beneficial, and which are just opportunists. The successful development of coral probiotics could see corals treated to survive bleaching or disease events. Topical antibiotic treatment is already being trialled amid the ongoing spread of stony coral tissue loss disease in the Florida Reef Tract (Neely *et al.*, 2020). It should be noted that while the stakes are high to ensure coral reefs

persist into the future, there are very real risks involved in interfering with the natural microbiomes of corals. As was found through the current project, coral microbiomes were very sensitive to disturbance, instigated even just through fragmentation and maintenance without antibiotic treatment (Chapter 4). Further stringent laboratory and controlled field experiments are required to improve our understanding of the coral microbiome and its potential for manipulation before any measures can be rolled out on reefs.

Most scientists understand that in the best case scenario, these active interventions can only do so much to buy time in the face of rapid environmental change, and in the worst case scenario, could upset the natural reef ecosystem balance. As such, we must continually question the ethics of actively intervening in the natural world (Sweet *et al.*, 2017a). Potential deleterious ramifications and long term consequences could include impacts on the natural biodiversity, such as genetic bottlenecks caused by selecting for certain traits, or the introduction of invasive non-native coral genotypes or microbial associates with coral transplants or farmed corals. With all the active intervention options available comes the caveat that these should be considered as a last resort. Of course, the main focus should be to provide evidence and put pressure on governments and global corporations, and shift public perceptions to drastically cut carbon emissions immediately. However, should the coral reef crisis become so dire that rapid active intervention is needed, it would be best to be prepared with the scientific basis and understanding of all options to hand.

Collectively, the research contained in this thesis has contributed to the growing body of knowledge on corals living in marginal habitats, in particular mangrove habitats. While findings show the potential of coral microbiomes to rapidly reorganise based on habitats with different environmental conditions, they raise further questions on the functionality and potential adaptive advantage of coral microbial symbionts. It is hoped that corals at the extreme can continue to teach us how corals might survive the challenging environmental conditions to come.

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Appendix I: Thermal performance of corals living in marginal habitats

Summary

The response of any organism to climate change depends on how its physiological performance varies from its optima toward extreme environmental conditions. This pilot study explored the thermal optima and thermal tolerance limits of reef-building corals from typical fore-reef habitats and marginal mangrove habitats around Hoga Island, Indonesia (Central Indo-Pacific Ocean) and Curieuse Island, Seychelles (Western Indian Ocean). Corals from the families Merulinidae (*Dipsastraea* cf. *pallida*, *Favites chinensis, Platygyra verweyi, Goniastrea edwardsi* from Indonesia), and Acroporidae (*Acropora* cf. *gemmifera* and *Acropora cf. digitifera* from the Seychelles), were subjected to temperatures ranging from 20°C to 38°C to capture their thermal performance. Differences in thermal performance of corals from reef and mangrove habitats were small, equating to approximately 1°C differences in cardinal temperatures such as optimum temperature for productivity (T_{optP}) and respiration (T_{optR}). The acute heat stress assays developed show utility for rapid testing of coral genotypes from different environments. However, higher replication is required to draw conclusions on the thermal performance of corals from reef and mangrove habitats.

A1.1. Introduction

Thermal performance underpins ecology; thermal optima and thermal performance limits define where organisms can live (i.e. their range limits and distribution patterns). Every organism has an optimum temperature at which it will thrive, and either side of this optimum,

Box A1.1. Glossary of thermal performance parameters (Kingsolver & Buckley, 2017)

T_{opt} – optimal temperature 'optimal temperatures are greater in systems where mean environmental temperatures are higher (and less variable)'

T_{optP} – optimal temperature for primary productivity

T_{optP} – optimal temperature for respiration

T_{br} – thermal breadth 'thermal breadths are wider in systems where environmental variation is greater'

T_{u} – upper thermal limit

CT_{max} - critical thermal maximal temperature – 'the threshold temperature at which an organism 'fails' an assay of performance (e.g. body posture or righting response, locomotory activity, neuromuscular control, survival)'

performance will decrease. This, in turn, defines where an organism can live. With climate change, areas that may have once been optimal for certain species may become sub-optimal, and with this, organisms will either have to move, or their populations will decline due to reduced fitness, or mortality (Kingsolver & Buckley, 2017). Corals are sessile organisms and cannot therefore move habitat as temperatures change. Therefore, they must either develop coping mechanisms, or their populations will decline, and they may eventually become extinct (Coles & Brown, 2003b; Byrne *et al.*, 2019).

To understand how organisms respond to temperature (or any change in environmental condition), fitness would ideally be measured directly. However, fitness itself is difficult to assess as it requires multi-generation studies, which, for long-lived organisms, are not practical. Fitness can instead be inferred from correlated measures of an organism's performance such as metabolism, growth rate, or other biological rate processes and functional traits (Huey & Stevenson, 1979). Thermal performance curves (TPCs) illustrate the effects of temperature on such performance traits (Baker et al. 2016). Commonly studied response measures for TPCs include functional performance traits (e.g. fecundity, growth, metabolic rate, and running speed), physiological processes (e.g. heart rate, nutrient uptake, carbon fixation, photoacclimation), and biochemical processes (e.g. enzyme activity; Schulte et al. 2011). This study focusses on the metabolic processes of photosynthesis and

respiration, as measured by changes in oxygen evolution and consumption by the coral holobiont.

Most previous studies into the effects of thermal stress on corals involve laboratory-based temperature ramping (reviewed in McLachlan *et al*., 2020). In these experiments, it is difficult to disentangle whether a coral's response is due to the current thermal stress or accumulated stress from a temperature inflicted several days prior. This co-linear relationship between temperature and time was one of the problems associated with the heat-ramping study presented in Chapter 2, in addition to a strong effect of aquaria acclimatisation. The effects of time-scale and cumulative heating on TPCs and thermotolerance is seldom considered in such experiments but can have large impacts on performance (Kingsolver & Buckley, 2017).

Corals are unique in that they are clonal organisms so can be fragmented to test the effects of temperature on the same genotype while avoiding double exposure and potential heat-hardening or cumulative stress to individual fragments. As such, acute heat stress assays were trialled here on individual fragments from the same coral colonies to build thermal performance curves for coral species found in both reef and mangrove habitats on either side of the Indian Ocean.

A1.2. Methods

A1.2.1. Coral collection

The thermal performance of corals from mangrove and reef habitats in two bioregions, the Western Indian Ocean and the Central Indo-Pacific Ocean, were investigated. Contrasting fore-reef and mangrove sites in the Western Indian Ocean were both located within Curieuse Marine National Park (CMNP), Seychelles (Fig. 1.6). Three colonies of *Acropora* cf. *gemmifera* were collected from the fore-reef site (Home Reef; 4° 17' 05.1" S, 55° 44' 07.6" E)

and the mangrove site (Turtle Pond; 4° 17 '12.9" S, 55° 43' 49.1" E) in May 2018 and three colonies of *Acropora* cf. *digitifera* were collected in 2019. Reef and mangrove sites in the Central Indo-Pacific were located within the Wakatobi Marine National Park, Southeast Sulawesi, Indonesia (Fig. 1.6). Three colonies each of four different merulinid coral species were collected from the reef site (Buoy 2; 5° 28' 31.2" S, 123° 45' 32.0" E) in July 2018. These were identified as *Favites chinensis*, *Platygyra verweyi*, *Goniastrea edwardsi*, and *Dipsastraea pallida* (formerly known as *Favia pallida*). Three colonies of *Dipsastraea* cf. *pallida* were also collected from the mangrove site (Langira; 5° 28' 41.1" S, 123° 43' 17.4" E). Coral colonies were fragmented upon collection to ensure that the same fragments were not subjected to thermal stress more than once (which could lead to heat-hardening). The thermal regimes of reef and mangrove environments was characterised, as detailed in previous chapters, using HOBO Pendant® Temperature/Light 64K Data Loggers (Model UA-002-64, ONSET, USA) programmed to record temperature every 15 minutes over a year.

A1.2.2. Thermal performance

The metabolism of corals from fore-reef and marginal mangrove habitats was measured over a range of temperatures from 20°C to 38°C to capture their thermal performance. The portable and cost-effective respirometry chamber set-up with heating and cooling capability is detailed in Chapter 2 (Fig. 2.4). Briefly, net primary productivity (NPP) and respiration (R) were measured by change in dissolved oxygen (DO) concentration during incubation of coral fragments in light and dark conditions, respectively. Gross primary productivity (GPP) was calculated by adding R (oxygen consumption) to NPP (net oxygen evolution). Productivity to respiration ratios (P/R) were calculated by dividing GPP by R. Every coral fragment (n = 3 per assay) was tested at ambient temperature (the average local temperature for that time of year was between 28°C to 29°C), followed by an assay temperature (ranging from 20°C to 38°C) to assess the difference in metabolism of each fragment with temperature (i.e. the response ratio). Thermal performance curves were constructed based on the change in GPP

(△GPP), the change in R (△R), the P/R ratio, and also the change in P/R (△P/R) between ambient and assay temperature.

Measuring coral metabolism at ambient temperature before each assay temperature allowed comparison of the difference in rates (△GPP, △R) independent of the coral's identity and surface area. Constructing a thermal performance curve based on the difference between assay metabolism and ambient metabolism meant that physiological performance better than ambient was represented by change in metabolism values greater than 1 (△ > 1), whereas performance worse than ambient was represented by △ < 1. Measuring P and R at ambient temperature twice, in succession, provided a control for the effect of time corals were kept in aquaria (no effect of time spent in aquaria would be represented as △ = 0).

A1.3. Results & Discussion

A1.3.1. Thermal performance curves

Corals from the thermally-variable Turtle Pond mangrove in the Seychelles naturally experience temperatures ranging from 25-35°C (Fig. 3.2).

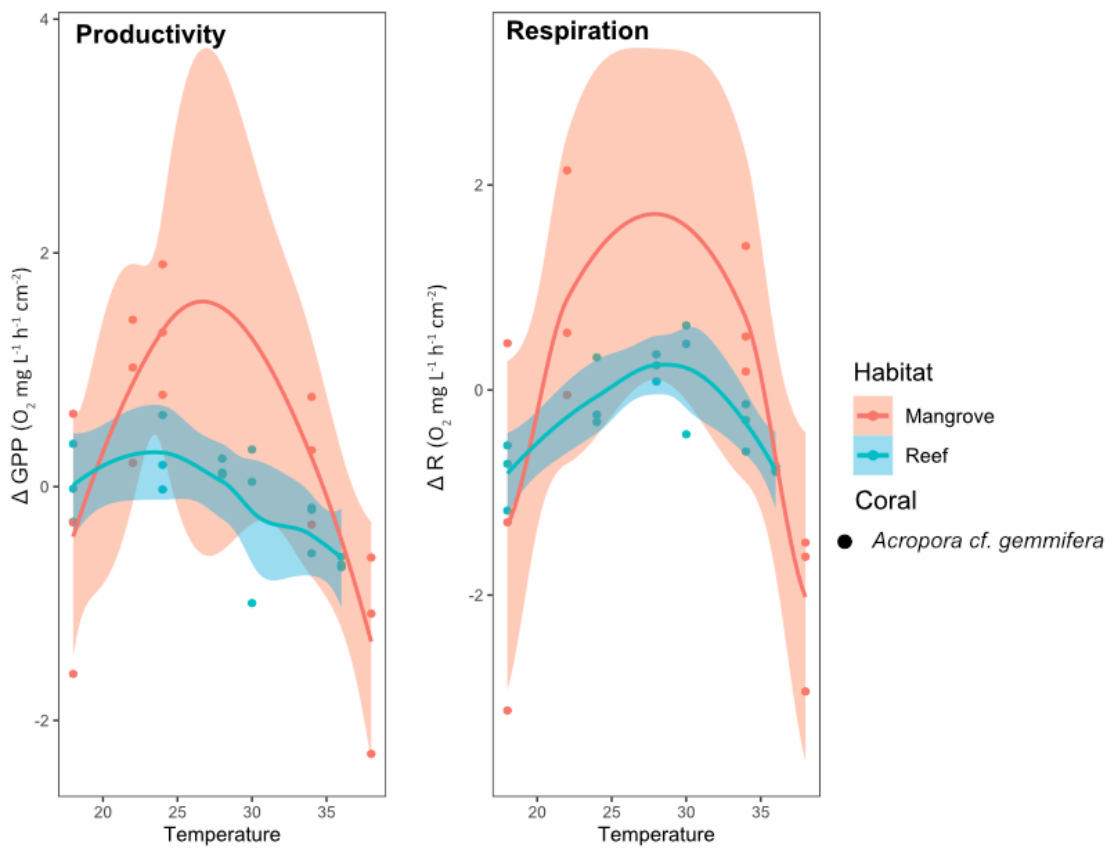

Figure A1.1. Thermal performance curves of *A. gemmifera* from mangrove (orange) vs. reef (blue) habitat. Values based on change in metabolic rate between ambient and assay temperature. Coloured shading represents ± SE of locally estimated scatterplot smoothing (loess – a type of local regression).

The thermal performance curves for *Acropora cf. gemmifera* from different habitats appear extremely different, suggesting they have adopted different metabolic strategies. The steep slope of the thermal performance curve for mangrove-origin *A. cf. gemmifera* (Fig. A1.1 orange) suggests its metabolism is temperature specialised. Whereas the shallower slope of reef-origin *A. cf. gemmifera* (Fig. A1.1 blue) suggests it has adopted a generalist approach with a lower thermal optimum. However, it should be noted that the error surrounding the thermal performance estimates is very high due to low replication (only three colonies tested per habitat), and missing data points for certain assay temperatures due to power outages.

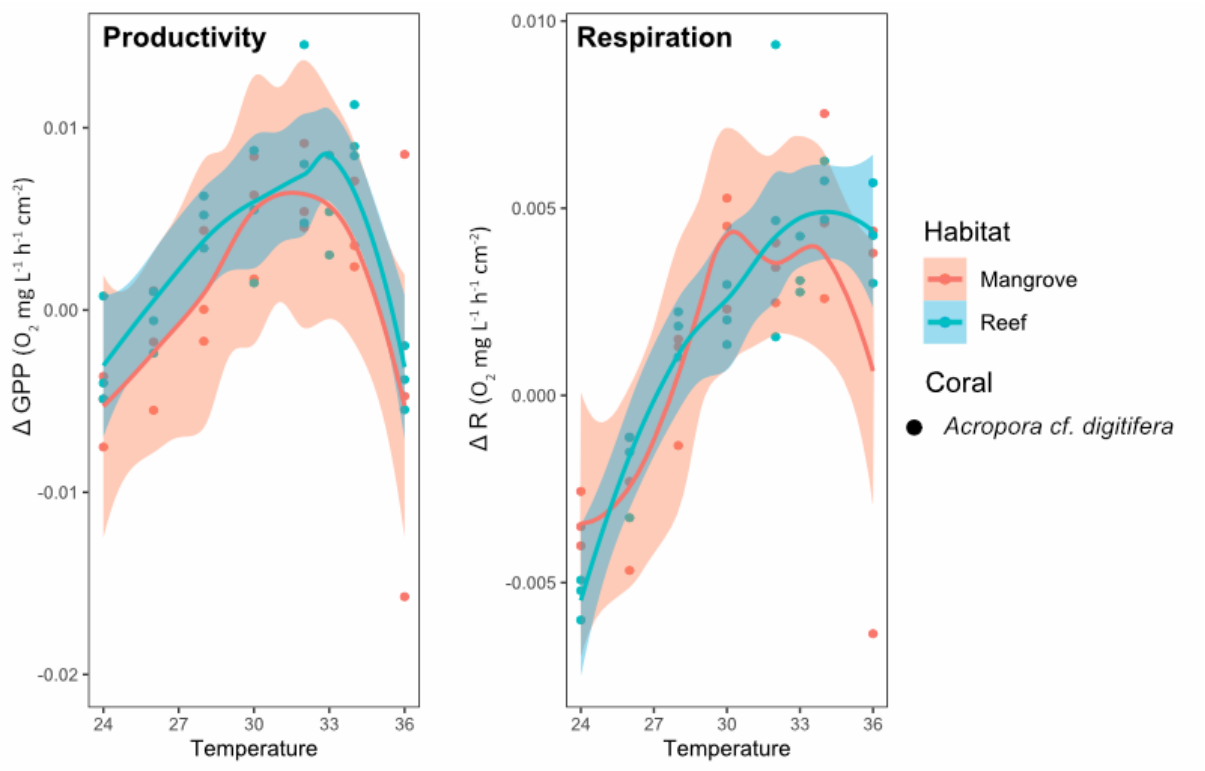

Figure A1.2. Thermal performance curves of *A. digitifera* from mangrove (orange) vs. reef (blue) habitat. Values based on change in metabolic rate between ambient and assay temperature. Coloured shading represents ± SE of locally estimated scatterplot smoothing (loess – a type of local regression).

Thermal performance curves constructed for *Acropora* cf. *digitifera* collected and tested in May 2019 surprisingly show that reef corals had marginally higher thermal optima (T_{optP} and T_{optR}) than mangrove corals. Though again, the error for these estimates is large and overlapping (Fig. A1.2). The similarity in thermal performance of mangrove and reef corals tested here despite large differences in the thermal regimes of their habitats (Fig. 3.2) could be explained by the recent mass-bleaching observed on the reef around Curieuse Island in 2016 (Gardner *et al.*, 2019). The bleaching and subsequent death of many heat-sensitive branching corals (Fig. 3.4) meant that *Acropora* sampled on the reef in 2019 were likely either heat-tolerant survivors of the mass-bleaching episode or new recruits from more tolerant genetic stock. Thus, it would be very interesting to study the thermal performance of corals from different habitats before and after a mass-bleaching event.

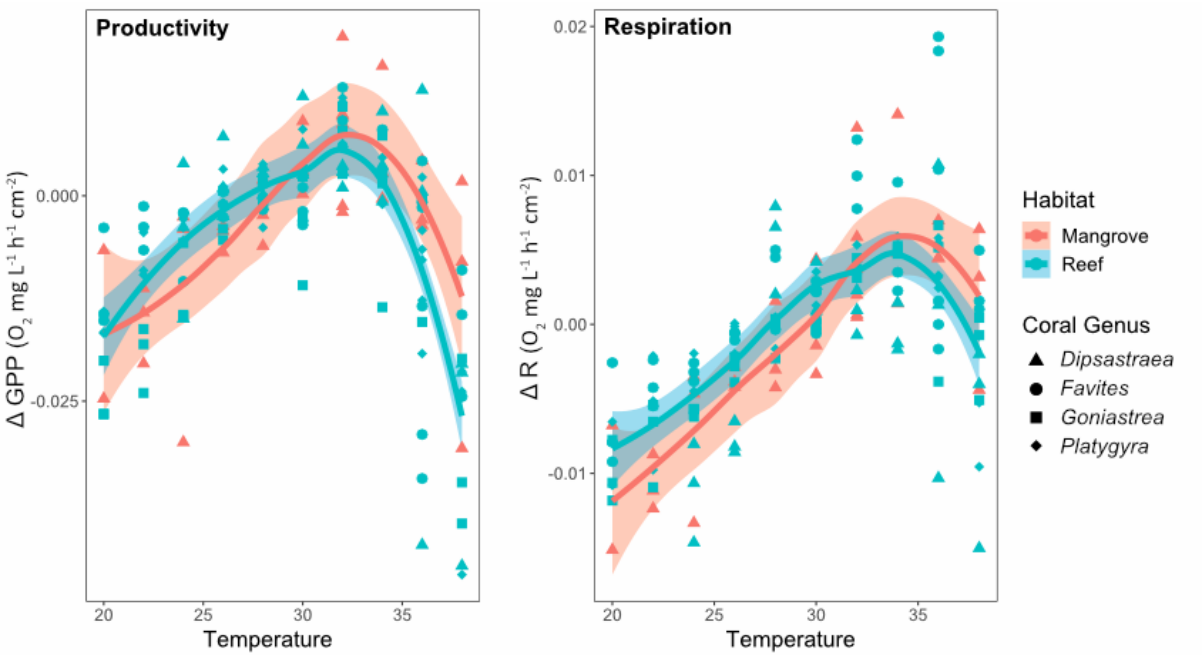

Figure A1.3. Thermal performance curves of family Merulinidae corals from mangrove (orange) vs. reef (blue) habitat. Values based on change in gross primary productivity (GPP) and respiration (R) between ambient and assay temperature. Shapes denote coral species. Coloured shading represents ± SE of locally estimated scatterplot smoothing (loess local regression).

The thermal performance curves for corals of the family Merulinidae from Buoy 2 reef (*Favites chinensis, Platygyra verweyi, Goniastrea edwardsi*) compared with Langira mangrove (*Dipsastraea* cf. *pallida*) show a subtle difference in metabolic response to temperature based on habitat. *Dipsastraea* cf. *pallida* from Langira mangrove environment (Fig. A1.3 orange) appears to have a higher optimum temperature (T_{opt}) than other merulinids from Buoy 2 reef (Fig. A1.3 blue). While this difference may appear small, only 1°C differences in cardinal temperatures such as critical maximum can mean the difference between a coral surviving or perishing during a warm water anomaly event. The high thermal optima of the corals from the mangroves is unsurprising since the maximum temperature recorded over the course of a year in Langira mangroves was as high as 38°C (Fig. 4.2).

Thermal performance curves show promise in predicting responses of populations or species to climate change, but researchers should use caution when using TPCs and be critically aware of the limitations of their study design when extrapolating data. Very different predictions can be obtained from TPCs generated in acute temperature response

experiments (Sitch *et al.*, 2003) versus those generated following chronic thermal exposure (Deutsch *et al.*, 2008). Voolstra *et al.* (2020) recently compared results of short-term acute heat stress assays (18 hours) versus longer term heat-ramping (21 days) for the hard coral *Stylophora pistillata* from exposed and protected sites in the Red Sea. Researchers found that the short-term acute heat stress assays resolved genotype (between colony) differences which could have been hidden by acclimation effects in the longer heat-ramping experiment. Results from the same study also highlighted that photosynthetic efficiency was the only response parameter indicative of higher thermotolerance in corals from the protected site in both short- and long-term studies (Voolstra *et al.*, 2020). A key limitation of the pilot presented here was the limited number of response parameters measured, including only primary productivity and respiration rates, which may be more highly conserved than photosynthetic efficiency.

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Appendix II: Efficacy of antibiotic treatment

NB. Methods trialled here were previously developed in Greenwood BN (2016) The importance of coral-associated bacteria to *Porites cylindrica* and *Stylophora pistillata* facing thermal stress. MSc thesis, University of Essex.

Summary

This pilot study was conducted as methods development for Chapter 4: Coral microbiomes are highly sensitive to active interventions: bacterial communities respond rapidly to antibiotic treatment and translocation. Preliminary data showed an antibiotic 'cocktail' of ampicillin, streptomycin, and nalidixic acid was effective. The concentration chosen for further experiments was 100 µg ml^{-1} as this reduced the viable bacterial load without having deleterious effects on the coral host.

A2.1. Materials and methods

Bacteria associated with the coral host, *Pocillopora damicornis*, were experimentally manipulated through 24 h treatment of antibiotics. Antibiotic treatment vessels (300 ml volume) were dosed with a combination of ampicillin, streptomycin, and nalidixic acid at different final working concentrations of 0 (control), 50, 100, 200, 400 µg ml^{-1}. These antibiotics were chosen to target previously known coral-associated bacteria such as *Vibrio* spp. (Mills *et al*., 2013) and other proteobacteria (Bourne & Munn, 2005).

Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, inhibiting cell wall synthesis, and resulting in cell lysis. It is effective against both gram-positive and some gram-negative bacteria. Streptomycin inhibits protein synthesis and is effective against both

gram-positive and gram-negative bacteria. Nalidixic acid inhibits DNA gyrase, the enzyme involved in supercoiling of DNA, thereby preventing DNA synthesis (Sigma-Aldrich, 2016). It has previously been found to be efficient at killing coral-associated bacteria without deleterious effect on coral (Mills *et al*., 2013). Stock solutions of 100 mg ml^{-1} of each antibiotic were prepared and filtered through 0.22 µm sterile syringe filters (Minisart, Sartorius) into sterile Falcon tubes, kept refrigerated at 4˚C, except ampicillin which was dosed directly after preparation since it forms an unstable solution.

Bacterial abundance following 24 h antibiotic treatment was estimated using the most probable number (MPN) technique with 96-well plates. Coral chips of approximately 2 mm were removed from the coral nubbins using an ethanol-sterilised scalpel and crushed in 1 ml FASW using a pestle and mortar. The skeleton was allowed to settle for 15 minutes and the supernatant was used as crushed tissue slurry. The crushed tissue slurry was vortexed in a 1.5 ml Eppendorf tube until homogenised, then centrifuged at 1000 × g for 5 minutes to isolate the zooxanthellae. The supernatant of the crushed tissue slurry was carefully pipetted, avoiding the zooxanthellae pellet, and 20 µl from each sample dispensed into the first row of 96-well plates. The 96-well plates were processed under sterile laminar flow conditions with a laboratory robot (Gilson PIPETMAX) which filled the 96 wells with enriched seawater medium (Bacto Marine Broth, Difco), and performed ten-fold serial dilutions. The robot added 180 µl media to every well. To conduct the serial dilutions, the robot transferred 20 µl from each sample in the first row to each well in the second row before mixing a pipette volume of 100 µl three times and repeating to the last row. One column was left without crushed tissue slurry sample to act as a blank media control. Plates were incubated at 26˚C for 48 h. Viability of bacteria was assessed using a plate reader (FLUOstar Omega, BMG Labtech) which conducted readings at 600 nm (A_{600}). Final absorbance values were blank-corrected with wells containing media only. Wells with final absorbance values greater than 0.1 were considered positive for bacterial growth in subsequent MPN calculations. Calculation of MPNs from the 96-well plates was carried out in R version 3.2.2 using code

developed by Dr Etienne Low-Décarie, based on the computation of MPNs in the Bacteriological Analytic Manual of the U.S. Food and Drug Administration (Sutton, 2010).

A2.2. Results

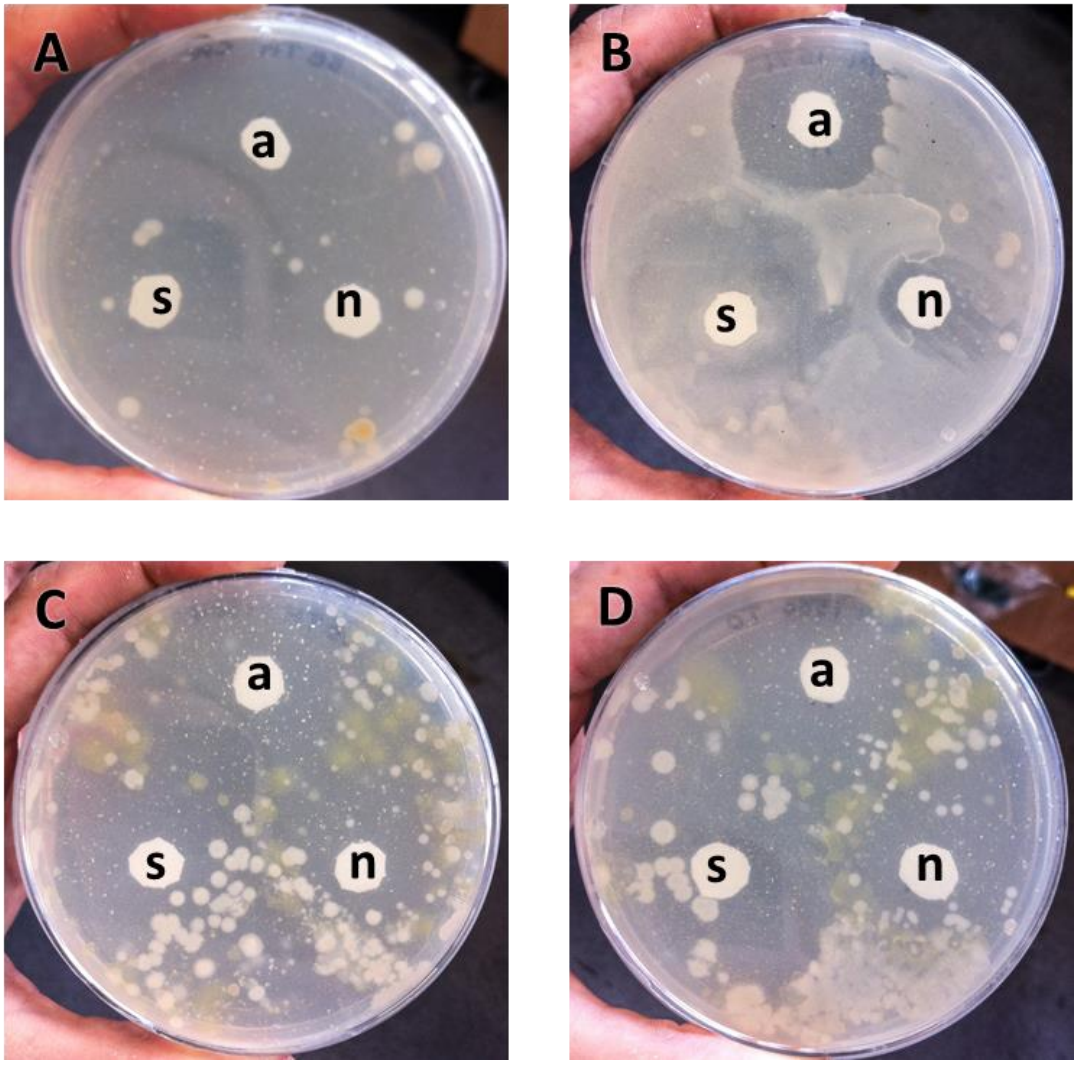

Figure A2.1. Agar plates of cultured bacteria from **A)** seawater, and **B-D)** coral tissue slurry, showing inhibition by antibiotics. **a**: ampicillin, **s**: streptomycin, **n**: nalidixic acid (Greenwood, 2016).

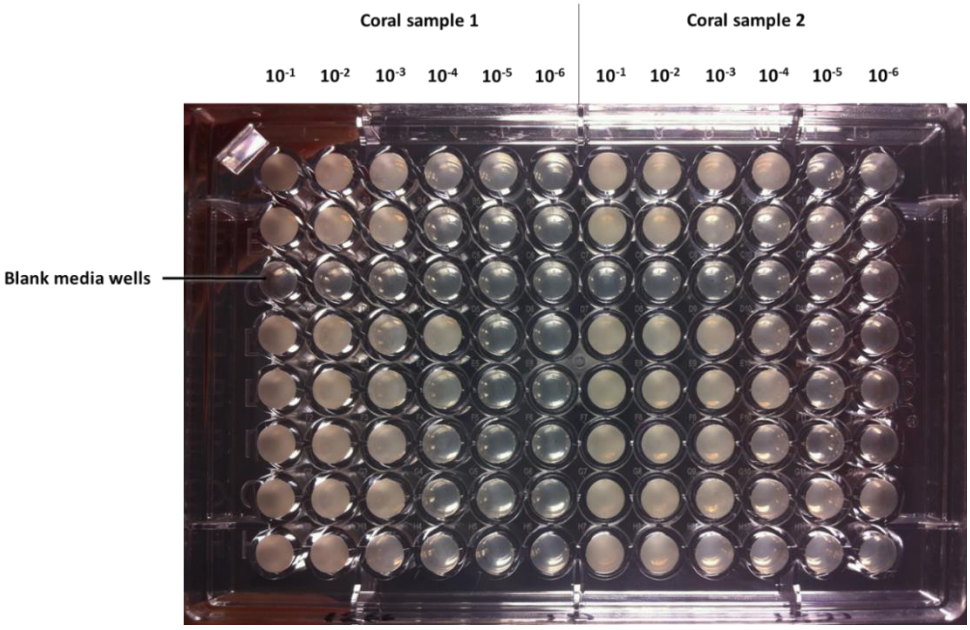

Figure A2.2. Ninety-six well plate of viable culturable bacteria (without antibiotic treatment) serially diluted 10^{-1} to 10^{-6} and grown for 48 h at 26˚C (Greenwood, 2016).

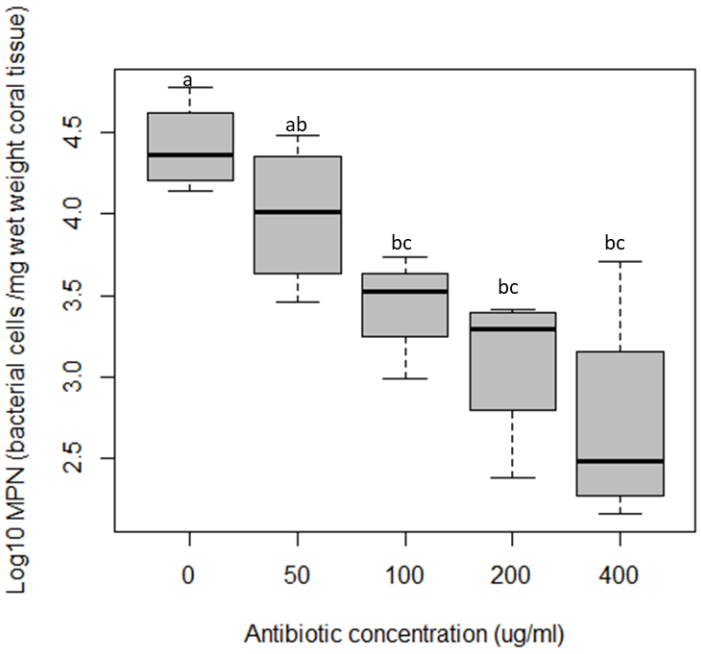

Figure A2.3. Viable counts of coral-associated bacteria determined by MPN estimation after 24h antibiotic treatment at 0, 50, 100, 200 and 400 µg ml^{-1} concentrations. Boxplots show median log10 MPN and interquartile range, error bars show range. Superscripts represent pairwise comparisons from Tukey's HSD test, following a one-way ANOVA.

There was a significant effect of antibiotic concentration on the most probable number of bacteria ($F_{(4,15)} = 9.273$, $P < 0.001$).

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