The unseen world of coral reefs: impact of local and global stressors on coral microbiome community structure

by

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Abstract

Diverse and abundant coral associated microbial communities may play a key role in coral resistance to and recovery from unwavering stressors currently threatening coral reefs worldwide. The composition and structure of the coral microbiome is integral to coral health as microbes can play beneficial (e.g. nutritional or protective) or negative (e.g. pathogenic or opportunistic) roles in the coral. To review the impacts of stressors on the coral microbiome, I compiled data from 39 studies, each tracking microbial community shifts in corals experiencing stress from climate change, pollution or overfishing. Stress was associated with shifts in coral microbial communities. I found that regardless of stressor, microbial alpha diversity increased under stress, with Vibrionales, Flavobacteriales and Rhodobacterales commonly found on stressed corals, and Oceanospirillales not as abundant on stressed corals. In addition, I used 16S rRNA sequencing to evaluate how local and global stressors affect the community structure of the coral microbiome for the two coral species, *Porites lobata* and *Montipora foliosa.* I monitored tagged coral colonies at two human disturbance levels (i.e. high and low), before and during a thermal bleaching hotspot at Kiritimati, Kiribati. Human disturbance, a bleaching hotspot, and coral species were all important drivers of coral microbiome community structure. My results suggest that human disturbance increases microbial alpha and beta diversity, although results vary between coral species, with *P. lobata* having more of a difference between disturbance levels. Similarly, bleaching increased beta diversity at low disturbance sites. Both human disturbance and thermal stress appeared to homogenize coral microbiomes between species and thermal stress appeared to homogenize communities between disturbance levels. Thus, both human disturbance and bleaching appear to stress the coral and destabilize its microbiome. However, intense thermal stress (i.e. 12.86 DHWs) appears to have a greater influence than human disturbance, probably due to corals responding to stressful conditions in a similar manner. In conclusion, my results highlight the impact of local and global stressors on coral microbiome community structure.

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Dedication

To my family, who inspire and challenge me.

Chapter 1- Introduction

Humans are altering ecosystems worldwide at alarming rates, with pervasive declines in biodiversity loss and local species abundances (Dirzo *et al.* 2014). In terrestrial ecosystems, humans are driving these changes through impacts like overexploitation, habitat destruction and introducing invasive species (Hoffmann *et al.* 2010). Humans are altering marine ecosystems through factors like climate change, overexploitation and land-based runoff (Halpern *et al.* 2008). Changes and loss of biodiversity, even at the microbial level (Delgado-Baquerizo *et al.* 2016), influence ecosystem change and functioning (Hooper *et al.* 2012) and have consequences for the ecosystem services that humans rely upon (Chapin *et al.* 2000; Worm *et al.* 2006). Halpern *et al.* (2008) suggests that no marine region in the world experiences no human impact, 41% of areas experience multiple human impacts, and the majority of coral reef ecosystems face medium high to very high human impact. These coral reefs are instrumental ecosystems to humans by providing an estimated 29.8$ billion dollars per year through fisheries, coastal protection, discoveries of new medicine and tourism (Cesar *et al.* 2003).

Coral reefs are one of the most diverse ecosystems on the planet, supporting an estimated ~830,000 multicellular species (Fisher *et al.* 2015), ranging from predatory sharks, herbivorous fishes, cryptic crustaceans to symbiotic microbes. These coral reefs are primarily founded by scleractinian corals (i.e. stony corals), with soft corals, algae, sponges and molluscs adding to the three-dimensionality of the reef. Scleractinian corals are comprised of coral polyps on a calcium carbonate skeleton, with polyps joining

together to form a coral colony. Corals contain the invertebrate animal itself and its associated microbes (i.e. *Symbiodinium*, bacteria, archaea, microscopic eukaryotes, fungi and viruses), all acting as an entire ecosystem, leading researchers to create the term the 'coral holobiont' (Rohwer *et al.* 2002). The most well studied microbe is *Symbiodinium*, the dinoflagellate alga that provides the coral animal with carbon from photosynthesis and receives protection and nutrients from the coral host (Baker 2003).

However, coral reefs worldwide are dramatically declining due to human induced threats like climate change, water pollution and overfishing. The severity of these declines varies across geographic regions. For example, the Caribbean has shown massive declines in hard coral cover by 80% over three decades with little variation between sub-regions suggesting synchrony among local stressors (Gardner *et al.* 2003). Furthermore, the Great Barrier Reef has demonstrated a 50% decline in coral cover over 27 years likely due to tropical cyclones, coral predation by crown-of-thorns sea stars, and coral bleaching (De'ath *et al.* 2012). Coral reef resilience (i.e. resistance to and recovery from a stress event) varies among regions and local environmental factors including coral species, temperature variation, nutrients, sedimentation, coral diversity, herbivore biomass, physical human impacts, coral disease, macroalgae, coral recruitment, and fishing pressure (McClanahan *et al.* 2012).

Local stressors, primarily water pollution and overfishing, currently threaten approximately 25% and 50% of coral reefs worldwide, respectively (Burke *et al.* 2011). Furthermore, a recent review suggests water pollution impacts 104 of 112 reef geographies worldwide (Wear and Thurber 2015). Water pollution, primarily from land-based runoff from humans (Szmant 2002; Fabricius *et al.* 2005), adds nutrients and

sediment to a primarily oligotrophic environment. Increased nutrients can increase coral susceptibility to bleaching (Wiedenmann *et al.* 2013) or increase algal growth, indirectly leading to coral mortality (Costa *et al.* 2000). Overfishing can also lead to increased algal cover on reefs through trophic cascades and phase shifts (Hughes 1994; Dulvy *et al.* 2004; Edwards *et al.* 2013), for example, through decreasing the number of herbivorous fishes that generally consume algae, resulting in an algal-dominated reef (McManus 2000).

Climate change, including ocean warming and acidification, is one of the leading stressors currently facing coral reefs (Hoegh-Guldberg *et al.* 2007; Doney *et al.* 2012). Increased CO_2 emissions in the atmosphere are increasing acidity and temperatures within coastal ecosystems. Acidification of coastal systems harms corals by decreasing calcification rates (Venn *et al.* 2013) and skeletal density, thus making corals more susceptible to erosion (Reyes-Nivia *et al.* 2013). The most well documented response to increased temperatures is coral bleaching. Coral bleaching occurs when the *Symbiodinium* are expelled from the coral tissue, leaving the translucent tissue layer over the white calcareous skeleton. When corals bleach there is a decrease in coral reproduction (Szmant and Gassman 1990) and growth (Goreau and Macfarlane 1990), and if *Symbiodinium* do not recolonize the coral tissue, the coral may die. Coral bleaching from high temperatures can lead to 95% (Wilkinson *et al.* 1999) or nearly 100% coral mortality (Brown and Suharsono 1990), and is a major concern for the future of coral reefs due to continuously warming oceans and an increase in ENSO (i.e. El Niño-Southern Oscillation) events (Cai *et al.* 2014). ENSO events are comprised of both El Niño (i.e. the warming phase) and La Niña (i.e. the cooling phase) with variation in

temperature and wind on the tropical eastern Pacific Ocean. Both 2015 and 2016 broke records as the warmest year, directly harming coral reef ecosystems through intense coral bleaching. For example, the northern 'pristine' region of the Great Barrier Reef lost ~67% of shallow water corals over 8-9 months in 2016 (ARC 2016). Subsequent stress events from increased temperature may enhance a corals ability to withstand increasing temperatures, however, with near-future temperature estimates continuously increasing, this bleaching protection may be lost and thus accelerate the rate of coral reef decline (Ainsworth *et al.* 2016).

With worldwide declines of coral reefs and recent technology advances, mainly in DNA sequencing and microscopy (Vega-Thurber *et al.* 2009; Bayer *et al.* 2013), researchers are now considering how microbes play a role in coral resilience to stress (Ainsworth and Gates 2016). The high diversity and abundance of microbes on corals was discovered in 2002 (Rohwer *et al.* 2002) and has been demonstrated to play a role in coral thermal tolerance (Gilbert *et al.* 2012) and eutrophication adaptation (Jessen *et al.* 2013). Researchers generally term the microbes associated with corals the 'coral-associated microbes' or the 'coral microbiome (i.e. the assemblage of microbes with the coral (bacteria, archaea and protists)) (Ainsworth *et al.* 2010). The microbiome plays varying roles for the coral host from nitrogen fixation (Lesser *et al.* 2004; Olson *et al.* 2009; Lema *et al.* 2012), sulphur cycling (Wegley *et al.* 2007; Raina *et al.* 2009), producing antibiotics to defend the coral from pathogens (Rypien *et al.* 2010; ElAhwany *et al.* 2013), or preying on pathogens in the coral mucus (Welsh *et al.* 2016). However, microbes can also harm the coral through bleaching (Kushmaro *et al.* 2001) or disease (Sutherland *et al.* 2011).

Thus, the community structure and assemblage of the coral microbiome is imperative to coral health and survival. Researchers have demonstrated that microbial alpha diversity increases with increasing sea water temperatures (Lee *et al.* 2016), potentially due to invading opportunistic or pathogenic taxa. Additionally, a new metric used by coral reef microbiologists is microbial beta diversity, which evaluates coral to coral variation (i.e. differences in the microbial assemblage between coral colonies). Zaneveld *et al.* (2016) suggested that an increase in microbial beta diversity within the coral indicates the stressed coral is unable to regulate its microbiome.

The work here seeks to understand the impact of both local and global stressors on coral-associated microbial community structure. My collaborators and I (1) review the current literature on studies that evaluate microbial community structure in corals under the three main stressors currently threatening coral reefs (i.e. climate change, water pollution and overfishing), and (2) observe and record the microbial community structure within corals on Kiritimati, Kiribati under stress from local human disturbance and a bleaching hotspot during the 2015/2016 El Niño.

In Chapter 2, we review the peer-reviewed literature for studies that evaluated the impact of climate change (i.e. ocean acidification or thermal stress), water pollution or overfishing on the coral microbiome. I compile trends from 39 studies and evaluate differences in microbial diversity and taxa. Overall, we demonstrate that microbial diversity tends to be higher in corals under stress and the bacterial taxa Vibrionales, Flavobacteriales and Rhodobacterales are often more abundant whereas Oceanospirillales are less abundant, in corals under stress. By synthesizing general responses of the coral

microbiome to stressors, we provide insight into an understudied, but integral, community found in coral reefs.

In Chapter 3, we empirically evaluate the influence of human disturbance and intense thermal stress on coral microbiome community structure using scientific diving and 16S rRNA sequencing of coral colonies at Kiritimati, Kiribati. This work provides the first observational study monitoring the coral microbiome across differing levels of human disturbance before and during a thermal stress event within two coral species (i.e. *Porites lobata* and *Montipora foliosa*). We demonstrate that human disturbance, thermal stress, and coral species all drive coral microbiome community structure with the general trend of increased alpha and beta diversity in the coral microbiome under both human disturbance and intense thermal stress. Our results suggest that both local and global stressors impact coral reefs down to the microbial level.

Together, these chapters aim to provide insight into how local and global stressors, known to dramatically influence the macroscopic communities on coral reefs, influence the microbial communities. Our work contributes to the rapidly evolving field of coral microbiology and suggests stressors worldwide are influencing the coral microbiome, potentially with negative consequences for the coral host.

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Chapter 2- Synthesizing responses of coral-associated microbial communities to local and global stressors

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2.1 Introduction

Coral reefs support an estimated ~830,000 multicellular species (Fisher *et al.* 2015) and contribute 29.8$ billion directly to the human economy annually (Cesar *et al.* 2003). Global threats like climate change, and local threats like water pollution and overfishing are known drivers of the drastic declines in these biodiversity hotspots and were recently shown to interact and influence communities at the microbial level (Zaneveld *et al.* 2016). For example, the hallmark of this deterioration is the Caribbean, where corals declined in cover by 80% over the last three decades (Gardner 2003). Yet, even the heavily protected Great Barrier Reef has lost coral cover with estimates reporting the current cover to be ~ 50% less than it was in 1985 (De'ath *et al.* 2012). Reefs will continue to deteriorate if these stressors are not fully mitigated; thus researchers have begun to focus on the mechanisms of coral resistance and resilience (Palumbi *et al.* 2014; van Oppen *et al.* 2015) and the role of microbes as sentinels in future reefs scenarios (Ainsworth and Gates 2016).

Even though recent research has focused on the role of microbiomes in coral adaptation (Gilbert *et al.* 2012; Glasl *et al.* 2016), coral reef management still largely ignores the role of microbial communities, with the exception of *Symbiodinium*, in coral resilience (McClanahan *et al.* 2012). Eukaryotic organisms harbour diverse microbial communities, essential to the evolution and adaptation of their hosts to environmental change (McFall-Ngai *et al.* 2013). The rapid evolution of culture-independent methods that began in the 1990's hastened the discovery of the diversity and abundance of coral-associated bacteria (Ritchie and Smith 1997; Rohwer *et al.* 2002; Ritchie 2006). As high throughput sequencing, microscopy, and microfluidics advanced, researchers gained better insight into the role and dynamics of coral-associated microbial communities at

larger scales and across larger time series (Garren and Azam 2011; Tout *et al.* 2015a; Welsh *et al.* 2016). The field of coral microbiology has grown enough over the past two decades to support the writing of a number of reviews on the topic, each with a different focus (Rosenberg *et al.* 2007; Ainsworth *et al.* 2010; Thompson *et al.* 2014). A recent manuscript discussed the onset and beneficial roles of coral-bacteria symbioses and how bacteria can influence reef responses to climate change (Sharp and Ritchie 2012). Here, we expand on and complement the body of available reviews by synthesizing results from 39 papers that evaluate how three key stressors threatening coral reefs (i.e. climate change, water pollution and overfishing) impact the coral microbiome. In addition, we provide hypotheses as to how the microbiome may provide corals with resistance to these stressors.

2.1.1 The Diversity of Coral-Associated Bacteria

Corals are diverse meta-organisms that contain not only the conspicuous algal partner *Symbiodinium* but also a microbiome composed of viral, bacterial, archaea, and other eukaryotic microorganisms. Within the coral surface mucus layer there are approximately 2-5 x 10^6 bacteria ml^{-1} and 0.1-3 x 10^7 viruses ml^{-1}; about 10 to 100-fold more than the water column (Wild *et al.* 2004; Marhaver *et al.* 2008; Nguyen-Kim *et al.* 2015). Host specific differences in microbiome composition suggest that most members of the microbiome are likely mutualistic (Ainsworth *et al.* 2015); thus many recent efforts have focused on identifying these bacteria and their specific metabolic roles in coral health (Table 2.1).

These abundant coral-associated bacterial communities are distinct from the surrounding habitat, containing taxa that drastically differ from free-living seawater

microbes (Carlos *et al.* 2013). In a meta-analysis of available 16S rRNA data for scleractinian corals, Blackall *et al.* (2015) found the most abundant taxa were Gammaproteobacteria (e.g. *Endozoicomonas*), followed by Alphaproteobacteria (e.g. *Vibrio* and *Serratia*). Bacteria can be conserved across species (Rohwer *et al.* 2002) and geography (Littman *et al.* 2009; Neave *et al.* 2016).

Bacterial community structure also varies spatially within individual corals. Similar to humans, compartmentalization of the microbiome generates distinct communities within the surface mucus layer, tissues, skeleton and gut (Sweet *et al.* 2010; Ainsworth *et al.* 2015). As compiled in Blackall *et al.* (2015), the number of bacterial OTUs that may be present varies depending on the microbial compartment in question. For illustration, tissue taxonomic richness can vary from ~50-500 OTUs (Ceh *et al.* 2012), while tissue and skeleton homogenized samples ranged from ~100-300 OTUs (Lee *et al.* 2012), and coral mucus ranged from ~250-3000 (Carlos *et al.* 2013).

Other aspects of coral biology also influence microbiome structure and function. For example, coral reproductive mode can determine what bacteria a coral larva will acquire. Coral-associated bacteria can be transferred vertically from parent to larva (Sharp *et al.* 2012) or they can be horizontally acquired from the environment (Apprill *et al.* 2009; Sharp *et al.* 2010), including when adult resident corals release bacteria (e.g. *Altermonas sp.* and *Roseobacter sp.*) as a by-product of broadcast spawning (Ceh *et al.* 2013b).

2.1.2 Are Coral Microbiomes Unusually Diverse?

Corals are sometimes referred as ‘highly’ diverse meta-organisms. Yet this is a somewhat subjective statement that likely has arisen when coral microbiomes are

compared to other well studied mutualistic symbiotic model systems that are highly canalized (Dubilier *et al.* 2008). It is now well accepted that microorganisms colonize most marine species, yet a systematic comparison among marine organisms is currently lacking. For example, sponge tissues contain between 10-1000 bacterial OTUs (Bourne and Webster 2013), a species richness value well within the range for corals. A recent assessment of tropical reef algal microbiomes also demonstrates that algae contain even more diverse bacterial communities than corals (Barott *et al.* 2011). The number of bacterial OTUs in corals can range up to 10^2-10^4 compared to 10^1-10^3 for sponges and 10^2 for *Hydra* (Blackall *et al.* 2015), although as just discussed these diversity estimates vary across species, habitat, and host compartment. With these context dependent numbers, it is thus difficult to say whether corals have a higher diversity of microbial taxa than other marine species. For example, Hester *et al.* (2015) compared the tissue bacterial communities of the corals *Acropora hyacinthus*, *A. rosaria*, and *Porites lutea* to crustose coralline algae (CCA) and turf algae. Turf and CCA each exhibited overall higher numbers of OTUs (18926 and 9559) than the three coral species (951, 2331, 4018). Furthermore, coral microbiomes range from hundreds to thousands of OTUs (902, 2188, 3662) while algal microbiomes were an order of magnitude higher (8856, 18065). Similarly, Barott *et al.* (2011) found that algal microbiomes were generally more diverse overall than those in corals. For example Shannon diversity values ranged from 2.84-4.51 for corals, compared to 6.22-7.82 for four types of algae (*Dictyota bartayresiana*, *Halimeda opuntia*, turf algae, and CCA). Therefore, more comparisons are needed to determine if corals actually have highly diverse microbial communities.

Additionally, when comparing the composition and dynamics of the coral microbiome to other marine hosts, it is important to differentiate between stable and sporadic members of the community. It is likely that stable members play more important roles in promoting the health and longevity of the host while sporadic members might play negative and antagonistic roles in the system. Stable microbes should exhibit consistent relative abundances in the host relative to the sporadic members who will vary in their prevalence and relative abundance among individuals of the same host (Hester *et al.* 2015).

Additionally, researchers evaluate the "core" microbiome at various levels of stringency of prevalence (e.g. 100%, 75%, or 50% presence in samples). This prevalence-based metric has been used in many instances to infer which members of a coral's microbiome are mutualistic or opportunistic. In an evaluation of the core coral microbiome (i.e. phylotype presence in 30% of the samples), *Acropora granulosa's* core microbiome consisted of 159 out of 1508 phylotypes, *Leptoseris spp.* 204 out of 1424, and *Montipora capitata* 350 out of 1433 (Ainsworth et al. 2015). Conversely, Hester *et al.* (2015) found a high number of stable members to sporadic microbes: *A. hyacinthus* (stable = 902, sporadic=49), *A. rosaria* (stable = 2188, sporadic= 143), *P. lutea* (stable = 3662, sporadic=356). Importantly, most of these core microbiome members were highly diverse yet found in very low relative abundance compared to the entire community. Thus it is important to consider rare microbiome members, as these may be the beneficial resident members. In another longitudinal study from three coral species from South Florida evaluating the core based on a >95% prevalence score, the core coral mucus microbiome consisted of 13 bacterial orders (Zaneveld *et al.* 2016).

2.1.3 Beneficial Roles of Coral Bacteria

Different coral-associated bacteria are hypothesized to play varying roles in coral development, health, nutrition and survival (Table 2.1). For example, diazotrophs (i.e. nitrogen-fixing bacteria) form species-specific associations with corals and may provide limiting fixed nitrogen to the algal partner of corals, *Symbiodinium,* and to the coral animal itself. For example, *Cyanobacteria* encoding nitrogen fixing enzyme genes were found to coexist with *Symbiodinium* in coral host cells (Lesser *et al.* 2004; 2007; Lema *et al.* 2012). In the early life stages of corals, bacteria provide nitrogen directly to the coral larva's *Symbiodinium* (Ceh *et al.* 2013a) and potentially to the coral larva itself (Lema *et al.* 2014). In a metagenomic study of the coral *Porites astreoides*, both nitrogen fixing and sulphur cycling genes were found and attributed to the coral-associated bacteria (Wegley *et al.* 2007), suggesting bacteria may provide these compounds to the coral holobiont. In addition, through sequencing and culturing, Raina *et al.* (2009) demonstrated the sulphur cycling potential of coral-associated bacteria. Zhang *et al.* (2015) found that coral-associated microbial communities contribute to carbon, sulphur, nitrogen and phosphorous fixation, metal homeostasis, organic remediation, antibiotic resistance and secondary metabolism.

Coral-associated bacteria may also defend the coral against potential pathogens by providing antimicrobial activities. Approximately 20% of cultivable isolates from *Acropora palmata* demonstrated antibiotic activity against other strains and pathogens (Ritchie 2006). These antagonistic interactions can help defend the coral from potential pathogens. Nearly 70% of culturable isolates from *Montastrea annularis* were inhibitory in Burkholder agar diffusion assays and 11.6% inhibited the growth of the known coral pathogen, *Vibrio shiloi* (Rypien *et al.* 2010). Isolates from the soft coral, *Sarcophyton*

glaucum, inhibited the growth of four coral pathogens and three fungi (ElAhwany *et al.* 2013). Zaneveld *et al.* (2016) found that under increased algal cover and increased temperatures, the relative abundances of Actinobacteria (i.e. antibiotic producers) decreased while opportunistic Proteobacteria increased within the coral microbiome, suggesting that opportunists can take advantage of the absence of inhibition. Additionally, 8% of native coral bacteria inhibited the growth of the pathogen*, Serratia marcescens*, with *Exiguobacterium sp.* inhibiting growth by 10-100 fold reductions in the coral mucus (Krediet *et al.* 2012). Concurrently, the coral pathogen, *Vibrio corallilyticus*, has antimicrobial activity of its own, suggesting that it may use inhibition to colonize the coral (Kvennefors *et al.* 2011). Not only do these bacteria inhibit coral pathogens, but also some bacteria actively prey upon these pathogens within the coral mucus (Welsh *et al.* 2016). Bacteria also play an important role in larval recruitment and settlement as shown in Sharp *et al.* (2015) where researchers identified an Alphaproteobacterium, *Roseivivax sp.* 46E8 that significantly increases larval settlement of *Porites astreoides*. Given that the roles played by coral-associated bacteria described above are vital to holobiont functioning, any disruption or destabilization can influence host fitness, survival and ecosystem functioning.

2.2 Responses of the Coral Microbiome to Stressors Threatening Coral Reefs

For this review, we synthesized 39 studies that examined how coral microbiomes respond to anthropogenic stressors. Over half of studies focused on climate change (n=22), almost one quarter focused on water pollution (n=10), and only a small proportion addressed overfishing (n=3) or more than one stressor at a time (n=4) (Figure 2.1). Over half of the studies were published in the last five years (Figure 2.1). Almost all

of the overfishing and water pollution studies occurred in the Caribbean, versus climate change studies that had a more global distribution of study sites (Figure 2.1).

The genera *Acropora* and *Porites* are the most represented corals within these studies, accounting for 44% of all corals evaluated (Figure 2.2a). Massive *Porites* species may be "stress-tolerant" corals, as they are slow growing and may be able to survive harsher environments. *Acropora* are considered to be 'competitive' corals, meaning that they are fast-growing and dominate reefs in productive environments; they are also the most sensitive to environmental change (Darling *et al.* 2012). As a result, there is a bias towards studying the effects of climate change on corals with competitive life history strategies (Figure 2.2b), specifically *Acropora* in Australia. We note here that because microbiologists have reported their data in different ways and at different taxonomic levels, we report bacterial taxa at all of the following levels for consistency: Phylum, Class and Order.

2.2.1 Stressors Increase Microbial Richness

Contrary to the species losses that often result from human impacts on macro-scale communities, the emerging evidence suggests that stressors commonly lead to an increase in bacterial richness or diversity within the coral microbiome community (i.e. ~60% of papers show increased diversity, Table 2.2). Invasion is the mechanism underlying these increases, with stress events appearing to disrupt the functioning microbiome and facilitating the invasion of non-coral microbes, thus increasing the overall number of microbiome members. For example, Morrow *et al.* (2012a) found that corals close to shore (i.e. closer to human disturbance) had higher bacterial diversity than corals more distant from shore, Meron *et al.* (2011) demonstrated the corals in lowered

pH had higher microbial diversity, Jessen *et al.* (2013) found that microbial diversity increased in all treatments of overfishing and eutrophication, and Zaneveld *et al.* (2016) found that contact with algae increased coral microbiome diversity. These results also are contrary to the patterns found in the human microbiome, in which stress lowers alpha diversity by allowing opportunistic and pathogenic taxa to dominate the community (Lozupone *et al.* 2012).

2.2.2 Stressors Alter Microbial Community Structure

In addition to richness increases, there is mounting evidence that stressors can induce changes to microbiome evenness and beta diversity. While species richness is a simple count of the number of species present, evenness takes into account the relative abundances of those species and beta diversity describes the variation among communities in a set of samples, in this case with individual corals representing communities (Anderson *et al.* 2011). For example, both temperature extremes and contact with macroalgae have been shown to increase microbiome beta diversity (i.e. increased variability in microbiome composition across coral colonies) (Zaneveld *et al.* 2016). Similarly, examining shallow coral reefs, Klaus *et al.* (2007) demonstrated that microbiome composition in polluted sites was both distinct from the control sites, and more variable from coral to coral than at the control sites. Microbes in polluted sites can be more pathogenic, as demonstrated by Mitchell and Chet (1975)'s study in which pollutants increased coral mortality except when antibiotics were added, suggesting a bacterial cause of death. In contrast, Lee *et al.* (2016) recently found that under high temperatures, bacterial communities shifted from being dominated by Betaproteobacteria

to Alphaproteobacteria and Verrucomicrobia, coinciding with a shift in mucus composition.

Changes in salinity can also impact the structure of coral associated microbial communities. For example, Röthig *et al.* (2016) found that the microbiome of hypersaline-treated corals shifted from a community dominated by a single OTU (Rhodobacteraceae*)* to a more even one in which *Pseudomonas veronii* was the most abundant taxon.

Although overfishing may seem unrelated to the coral microbiome, by decreasing herbivorous fish abundance, overfishing leads to reduced grazing pressure, increased macroalgae, and hence increased coral contact with macroalgae on reefs (Morrow *et al.* 2013). Macroalgae are hypothesized to outcompete corals via a variety of mechanisms including alterations to the microbiome (Smith *et al.* 2006; Morrow *et al.* 2012b), shading, abrasion and preventing coral recruitment (Jompa and McCook 2003) and allelopathic interactions (Rasher and Hay 2010). For example, macroalgal contact with the coral *Porites astreoides* caused multiple changes in the coral microbiome including increased dispersion, disappearance of a potentially mutualistic Gammaproteobacteria*,* changes in taxa abundance, the establishment of new taxa, and growth of algae-associated microbes within the coral (Vega-Thurber *et al.* 2012). Macroalgal contact has been shown to shift the coral microbiome to become more similar to the macroalgal microbiome (Morrow *et al.* 2013).

A counter example to the overall trend of stress-induced community shifts is provided by a study on *A. millepora* and *Seriatopora hystrix* microbiomes. These corals demonstrated stability in microbiome composition in the face of lowered pH together

with increased temperature simulating future climate condition projections. While *S. hystrix*'s microbiome did shift some, the changes were not statistically significantly ($p=0.058$), and the overarching take-home message was that corals demonstrated a more stable and robust microbiome compared to other key calcifying reef taxa such as foraminifera and crustose coralline algae (Webster *et al.* 2016).

2.2.3 Stressors Decrease the Abundance of the Putative Bacterial Symbiont, Endozoicomonas

Our analysis also found consensus that the bacterial order, Oceanospirillales, especially the genus *Endozoicomonas,* was consistently underrepresented in corals during stress events, especially during climate anomalies (Figure 2.3). This may be problematic for corals as *Endozoicomonas* is thought to be a beneficial symbiont for corals. Neave *et al.* (2016) and Bayer *et al.* (2013) used CARD-FISH and FISH probes, respectively, to reveal that *Endozoicomonas* was located deep within the coral tissues, suggesting an intimate association with coral hosts. Additionally, investigating the first cultivable *Endozoicomonas* from corals, Ding *et al.* (2016) suggested *Endozoicomonas montiporae* CL-33 helps corals under stress through preventing mitochondrial dysfunction and promoting gluconeogenesis. Additionally, researchers have proposed that *Endozoicomonas* plays a role in sulphur cycling (Neave *et al.* 2016), nutritional symbiosis (La Rivière *et al.* 2013) and protecting *Symbiodinium* from bleaching pathogens (Pantos *et al.* 2015).

This decrease in potentially beneficial taxa could not only threaten coral resistance to a stressor, but also coral resilience after the stressor is alleviated if *Endozoicomonas* does not return to its original abundance. In a study of natural volcanic

CO_2 seeps, *Acropora millepora* and *Porites cylindrica* contained significantly different microbial communities at sites with naturally reduced pH, mainly due to a 50% decrease of *Endozoicomonas* (Morrow *et al.* 2015). In another study *Endozoicomonas* was significantly reduced at low pH in *A. millepora* (Webster *et al.* 2016). Other symbiotic taxa in addition to *Endozoicomonas* are likely to decline as well under stress. At anthropogenic impacted reefs, the main coral symbiotic taxon in *Pocillopora verrucosa* (i.e. Endozoicomonaceae) and *A. hemprichii* (i.e. Altermonadales) declined in relative abundance (Ziegler *et al.* 2016).

2.2.4 Stressors Increase Opportunistic and Pathogenic Taxa in the Coral Microbiome

Stressed corals may have a lower ability to regulate their microbiome and thus have increases in potentially pathogenic and opportunistic taxa. Under all three stressors, the overrepresented taxa during stress were: Cyanobacteria, Flavobacteriales, Rhizobiales, Rhodobacterales, Rhodospirillales, Deltaproteobacteria (including Desulfobacterales and Desulfovibrionales), Altermonadales, Vibrionales, Pseudomonadales, and Enterobacterales (Figure 2.3).

Specifically, corals stressed by climate change (i.e. acidification and high thermal stress) had overrepresented Vibrionales, Cyanobacteria, Rhodobacterales, and Verrucomicrobia (Figure 2.3). In comparison, in a meta-analysis of 16S sequences from 32 papers, bleached corals had similar microbiomes to healthy corals but a higher proportion of two main taxa, *Vibrio* and *Acidobacteria* (Mouchka *et al.* 2010), but see (Koren and Rosenberg 2006; Salerno *et al.* 2011). An increase in Vibrionales under climate change stress is unsurprising as the cultivable *Vibrio* strain AK-1 was shown to

induce coral bleaching (Kushmaro *et al.* 1998) and Vibrionales are known to increase during thermal stress (Bourne *et al.* 2007; Frydenborg *et al.* 2013; Tout *et al.* 2015b).

Bleached corals have different bacterial communities than 'healthy' corals (Koren and Rosenberg 2008) . During a bleaching event in Australia, the coral-associated microbial community showed an increase in the expression of virulence genes (Littman *et al.* 2011). Correspondingly, during heat stress experiments, the pathogen *Vibrio coralliilyticus* increased in abundance by four orders of magnitude (Tout *et al.* 2015b). Yet, competition between pathogenic bacteria (i.e. *V. shiloi* and *V. coralliilyticus*) and native coral commensals is moderated by temperature within *Acropora palmata*, where high temperature favours pathogens (Frydenborg *et al.* 2013). Increasing temperatures correlate with virulence gene expression (Banin *et al.* 2003), coral lysing (Ben-Haim *et al.* 2003), and infection (Kushmaro *et al.* 1998; Ben-Haim and Rosenburg 2002) by coral pathogenic bacteria. This increase in *Vibrio* was shown to occur prior to visual bleaching signs (Bourne *et al.* 2007) and other shifts in taxa also occurred prior to visual bleaching (Lee *et al.* 2016), suggesting these changes in the bacterial community could forewarn which corals may bleach. This increase in Vibrionales within the microbiome may be regulated by a variety of factors including what *Symbiodinium* is hosted within the coral (Littman *et al.* 2010).

The increase in these pathogens may be due to temperature sensitive virulence cassettes, enhanced growth rates, or signals sent from the host. Thermally stressed corals increase production of the metabolite, dimethylsulphoniopropionate (DMSP) which is normally exuded by corals (Raina *et al.* 2013) and their symbionts (Steinke *et al.* 2011). It is hypothesized that DMSP is used by bacterial pathogens as a chemo attractant to

locate thermally stressed corals (Garren *et al.* 2014). Other stressors, such as increased temperature, nutrients, DOC, or pH, increased the expression of virulence sequences in the coral holobiont (Vega-Thurber *et al.* 2009).

Flavobacteriales and Rhodobacterales were overrepresented within corals stressed by water pollution (Figure 2.3). Similarly, coral microbiomes subject to overfishing pressures showed enrichment in Flavobacteriales, Pseudomonadales, Desulfovibrionales and Rhodobacterales (Figure 2.3). Rhodobacterales are fast growing opportunistic bacteria (Teeling *et al.* 2012) and have been found in both healthy and stressed corals (Meron *et al.* 2011; Sharp *et al.* 2012), potentially blooming under periods of stress when there is open niche space (Welsh *et al.* 2015). OTUs within Flavobacteriales were found to make up 27% of the OTUs associated with white band disease (Gignoux-Wolfsohn and Vollmer 2015). Thus these potentially pathogenic, opportunistic taxa may flourish when the coral is stressed and cannot regulate its microbiome. However, shifts in microbial community structure do not always indicate stressed corals. For example, Tracy *et al.* (2015) found *Orbicella faveolata*'s microbiome did not significantly shift when the host bleached. In contrast, *Gorgonia ventalina* did not bleach and showed persistent shifts during the thermal anomaly with shifts continuing even one year after the thermal event.

2.2.5 Climate Change, Water Pollution and Overfishing Increase Pathogens, Heterotrophs and Disease Related Sequences

Stressors can also increase pathogens, heterotrophs and disease on coral reefs. A metagenomic study evaluating a bleaching event in Australia found the coral-associated microbiome shifted from a community dominated by autotrophs to heterotrophs (Littman

et al. 2011). While coral calcification has been the central focus of ocean acidification, Vega-Thurber *et al.* (2009) and Meron *et al.* (2011) found that coral microbiomes exposed to lower pH were reminiscent of those associated with diseased and stressed corals, as they contained more Vibrionaceae and Altermonadaceae. Additionally, pH decreases significantly changed the microbial communities in *A. millepora* with the loss of Proteobacteria sequences associated with healthy corals while Gammaproteobacteria associated with diseased and stressed corals increased (Webster *et al.* 2012).

Conversely, Meron *et al.* (2012) found that coral-associated microbial communities did not undergo major shifts when transplanted to a natural lower pH environment, nor did they detect any microbial pathogens. This study was conducted in the field, therefore having no confounding factor of aquarium disturbance, thus suggesting that for these two coral species (i.e. *Balanophyllia europaea* and *Cladocora caespitosa*), reduced pH does not pose a significant threat to coral health.

Given that coral reefs are oligotrophic environments, added nutrients can dramatically influence ecosystem functioning and alter nutrient-sensitive microbial communities. Water pollution can directly add pathogens to coral reefs. In Florida, human sewage supplied a strain of *Serratia marcescens* (a common faecal enterobacterium) into reef water and corallivorous snails acted as a vector of *S. marcescens*, therefore inducing white-pox like diseases in *Acropora palmata*, *Siderastrea siderea* and *Solenastrea bournoni* (Sutherland *et al.* 2010). Furthermore, the addition of glucose or inorganic nutrients improved the survival of *S. marcescens* in *A. palmata* (Looney *et al.* 2010). Under this water pollution stress, microbial communities can have skewed abundances of heterotrophs to autotrophs (Dinsdale *et al.* 2008). As the proximity

and size of human population centers near coral reefs continues to grow, so does the likelihood of increased land-based runoff. Evidence continues to mount that corals living closer to shore have higher abundances of disease-related bacteria (Morrow *et al.* 2012a). Additionally, reefs impacted by human disturbance can have higher abundances of bacteria in the water column (Figure 2.4). Nevertheless, the coral microbiome can demonstrate resilience against water pollution stressors, for example, when coral fragments were transplanted under eutrophic aquaculture pens, the coral microbiome shifted towards known pathogens but showed no physical signs of disease and after 22 days the communities shifted back to their original state (Garren *et al.* 2009).

Given that overfishing can induce population declines and phase shifts on coral reefs (Hughes 1994; McManus 2000) that favour increases in algal cover, the relative dominance of algal versus coral cover is a major concern. Increases in algal interactions can influence both the water column microbiome bathing the corals (Morrow *et al.* 2013) and coral disease. Coral interactions with turf algae have been associated with an increase in pathogens and virulence genes (Barott *et al.* 2011). Moreover, algae may act as reservoirs for coral pathogens (Sweet *et al.* 2013) and thus enhance disease events. Algae harbour distinctly different microbial communities than corals (Barott *et al.* 2011; Vega-Thurber *et al.* 2012) and produce more DOC, thus increasing heterotrophic microbial growth in reef waters (Haas *et al.* 2011). Algae also produce dissolved organic matter (DOM) with a different chemical composition than coral-produced DOM. Algal DOM is enriched in dissolved neutral sugars (DNS) that selects for less bacterial diversity and microbiomes dominated by copiotrophic Gammaproteobacteria that typical carry increased quantities of virulence factors (e.g. Vibrionaceae and Pseudoaltermonadaceae).

Conversely, corals exude DOM that selects for high bacterial diversity dominated by Alphaproteobacteria and few representatives with virulence factors (e.g. Hyphomonadaceae and Erythrobacteraceae) (Nelson *et al.* 2013; Haas *et al.* 2016). Barott *et al.* (2012) propose that fleshy algae alter reefs by increasing bacterial respiration and allowing pathogenic invasions.

Overfishing also changes population sizes of fish and induces trophic cascades (Jackson *et al.* 2001), and thus influences the reef-associated microbial communities. For example, within the territory of the damselfishes, *Stegastes apicalis* and *S. nigricans*, there were 2-3-fold increases in potential coral pathogens in the microbiome and a higher prevalence of corals with blackband disease. These *Stegates* species exclude macroalgae and cultivate filamentous algae, providing a link among fish behaviour, coral pathogen reservoirs and coral disease (Casey *et al.* 2014). Furthermore, functionally diverse fish communities have been suggested to alleviate coral disease, and certain fishes (i.e. *Chaetodontidae*) may act as disease vectors (Raymundo *et al.* 2009). However, Cole *et al.* (2009) found that some damselfish actually slowed the progression of blackband disease. Furthermore, coral damage from abandoned fishing lines explained the differences between reserve and non-reserve areas in coral disease prevalence, with reserves having four-fold reductions of coral disease prevalence when compared to non-reserve sites (Lamb *et al.* 2015).

2.3 Evidence that Coral Microbiomes Mediate Host Resistance to Stressors

Some of the strongest evidence in support of the hypothesis that coral microbes protect their hosts against stressors comes from studies using antibiotics. For example, antibiotic treatment of thermally stressed corals caused tissue loss, significant declines in

photosynthetic efficiency (Gilbert *et al.* 2012) and increased coral susceptibility to *Vibrio shiloi* infection and bleaching (Mills *et al.* 2013), although see (Bellantuono *et al.* 2012). Furthermore, when corals were subjected to antibiotics and subsequently transplanted back onto the reef, they bleached and eventually died (Glasl *et al.* 2016).

Early investigations into the role of DSMP (i.e. dimethylsulfoniopropionate) cycling, nitrogen fixation and regulation by coral residents suggested that the coral microbiome likely plays an important role in coral resistance to stress. Bacteria implicated in sulphur cycling (e.g. *Endozoicomonas*, *Halomonas*) (Raina *et al.* 2009; Todd *et al.* 2010) may help corals acclimate to climate change by breaking down DMPS and thus protecting *Symbiodinium* from photosynthesis derived oxidative stress, as DMSP and its breakdown products act as antioxidants for marine algae (Sunda *et al.* 2002). As such, Pantos *et al.* (2015) demonstrated a negative correlation of bleaching bacterial pathogens with an increase in *Endozoicomonas*. However, Steinke *et al.* (2011) found that cultured *Symbiodinium* strains with known high thermal tolerance did not have higher intracellular concentrations of DMSP/ DMS and no sulphur degrading genes were found in the genomes of three marine symbiotic *Endozoicomonas* bacteria (Neave *et al.* 2014), thus demonstrating conflicting support for this hypothesis. Diazotrophs may play an important role in coral resistance to both climate change and water pollution. *Symbiodinium* depend on nitrogen for growth (Béraud *et al.* 2013) and diazotroph abundance increases with increasing seawater temperatures (Santos *et al.* 2014). However, the nuances of this relationship remain an active area of research as diazotrophs may in fact harm corals during heat stress by increasing the N:P ratio, destabilizing the symbiosis and increasing the threat of bleaching (Rädecker *et al.* 2015).

Furthermore, diazotrophs may play an important role in buffering the coral holobiont under water pollution by fixing nitrogen. In eutrophication experiments on *Acropora hemprichii*, nitrogen fixing and denitrifying bacteria increased in abundance with no significant changes in holobiont physiology (Jessen *et al.* 2013). However, in both water pollution and climate change, once a threshold is passed, and bottom up control is released, the holobiont may lose the beneficial association as diazotrophs thrive opportunistically. Finally, coral-associated bacteria may assist coral resistance to overfishing, specifically algal growth, as resident bacteria defend the coral from invasions (see "Beneficial Roles" above). There are multiple mechanisms that influence these microbial roles and as demonstrated above, conflicting evidence. We suggest these are key topics to better understand the microbial role in coral resistance to stress.

2.4 Conclusions, Considerations and Ways Forward

Bacteria are important members of coral holobionts and our understanding of how they contribute to reef health is rapidly evolving. Here we review studies on how microbial communities respond to environmental change, as well as how they may protect corals from stress. When stressors induce changes in bacterial communities, a loss of beneficial functions normally played for the coral may result. The composition of the coral-associated microbiome may provide insight for resource managers into which corals are most likely to successfully resist stressors. Studies suggest that stressed corals will have diverse microbiomes with an overrepresentation of Cyanobacteria, Flavobacteriales, Rhizobales, Rhodobacterales, Rhodospirillales, Deltaproteobacteria (including Desulfobacterales and Desulfovibrionales), Altermonadales, Vibrionales, Pseudomonadales, and Enterobacterales, while Oceanospirillales will be

underrepresented. Thus managers may be able to identify stressed corals due to the presence of these opportunistic taxa (Pollock *et al.* 2011) and increased microbiome diversity, even if the coral has not yet shown physical signs of stress or deterioration. As climate projections predict future conditions will increase disease susceptibility, pathogen abundance and virulence on coral reefs (Maynard *et al.* 2015), it is imperative that researchers focus efforts on understanding the coral microbiome. Promisingly, ongoing research suggests we may have the ability to increase coral adaptation to these stressors by modifying the coral-associated microbial community (i.e. viruses, bacteria, archaea and micro-eukaryotes) (van Oppen *et al.* 2015). Evidence is accumulating that researchers must continue to focus efforts on unraveling roles and mechanisms among bacterial taxa and the coral host's success in a continuously threatened environment.

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Tables

Table 2.1. Overview of the proposed beneficial roles of different coral-associated bacteria.

Role	Description	Example Taxa	Reference
Metabolism	Provide nitrogen to *Symbiodinium*	Cyanobacteria (potentially *Synenochocous* and *Prochlorococcous*)	Lesser *et al.* 2004
		Cyanobacteria	Lesser *et al.* 2007
	Provide nitrogen to coral larvae's *Symbiodinium*	*Altermonas sp.* and *Vibrio alginolyticus*	Ceh *et al.* 2013a
	Provide nitrogen to the coral larvae	Rhizobiales	Ceh *et al.* 2013a, Lema *et al.* 2014
	Provide nitrogen to *Symbiodinium* and the coral	Gammaproteobacteria, specifically *Vibrio sp*.	Olson *et al.* 2009
		Rhizobiales	Lema *et al.* 2012
	Provide nitrogen and sulphur to the holobiont	Unknown	Wegley *et al.* 2007
	Sulphur cycling	*Roseobacter, Spongiobacter, Vibrio, Altermonas*	Raina *et al.* 2009
	Cycling sulphur, carbon, nitrogen and phosphorous cycling to the holobiont, metal homeostasis, organic remediation, antibiotic resistance, secondary metabolism	Unknown	Zhang *et al.* 2015

Protection	20% of cultured bacteria had antibiotic activity against other strains and pathogens	*Photobacterium, Halomonas, Exiguobacterium, Bacillus, Altermonas*	Richie 2006
	~70% of culturable isolates from corals demonstrated inhibition to Burkholder agar diffusion assays	Vibrionales, Altermonadales, *Pseudoaltermonas,* Bacteroidetes	Rypien *et al.* 2010
	Cultured isolates from corals demonstrated inhibition of four coral pathogens and three fungi	*Bacillus, Psuedomonas*	ElAhwany *et al.* 2013
	Opportunistic Proteobacteria increased when Actinobacteria were below ~2.5% relative abundance	Actinobacteria	Zaneveld *et al.* 2016
	8% of coral commensals inhibited glycosidases (needed for growth) and of catabolic enzymes in a coral pathogen, *Serratia marcescens*	Exiguobacterium	Krediet *et al.* 2012
	Predatory bacteria consume the coral pathogens *Vibrio corallyticus* and *V. harveyii*	*Halobacteriovorax sp.*	Welsh *et al.* 2016
	Displayed antimicrobial activity against other coral cultured isolates	*Vibrio corallyticus, Pseudoaltermonas*	Kvennefors *et al.* 2011
Recruitment	Cultures and filtrates significantly increased larval settlement suggesting an extracellular factor	*Roseivivax* sp. 46E8 (Alphaproteobacterium)	Sharp *et al.* 2015

Table 2.2. Changes in microbiome diversity due to stress from climate change, water pollution and overfishing (+ =higher diversity, - =lower diversity, 0= no difference).

Stressor	**Specific Stressor**	**Changes in Diversity?**	**Direction**	
Climate Change	Thermal Stress	Higher diversity within a coral colony during bleaching compared to pre- and post-bleaching	+	Bourne *et al.* 2007
		Diazotroph diversity and richness significantly increased 3-fold with +2.5º and +4º C increases	+	Santos *et al.* 2014
		Diversity was significantly higher under heat stress (31º C)	+	Tout *et al.* 2015b
		Gorgonia ventalina microbial diversity was significantly lower during the warming event comparing to pre-warming, *Orbicella faveolata* was not significantly different to pre-warming	- 0	Tracy *et al*. 2015
		Richness and diversity increased with increasing temperatures	+	Lee *et al.* 2016
	Ocean Acidification	Increase in alpha diversity at lower pH	+	Meron *et al.* 2011
		No significant changes except alpha diversity significantly decreased in *Cladocora caespitosa* skeleton (two coral species, looking at skeleton and tissue)	- 0,0,0	Meron *et al.* 2012
		Porites cylindrica had lower	-,0	Morrow *et*

		alpha diversity at low pH while *Acropora millepora* showed no significant change		*al.* 2015
Water Pollution	Proximity to Humans	*Porites astreoides* and *Montastrea faveolata* had higher diversity at sites closer to shore (i.e. < 5km)	+,+	Morrow *et al.* 2012a
		Alpha diversity in *A. hemprichii* was significantly higher at sites impacted by sedimentation and sewage, *Pocillopora verrucosa* showed no significant difference. Both showed no significant different at wastewater outfall.	+,0,0,0	Ziegler *et al.* 2016
	High Salinity	Corals within the long-term salinity treatment increased in microbial richness 3-fold and diversity 10-fold	+	Röthig *et al.* 2016
	Oil Pollution	The number of bands increased after microcosm experiments exposed corals to 1ml and 20 ul of crude oil	+	Al-Dahash and Mahmoud 2013
Water Pollution & Overfishing	Eutrophication & Herbivore Exclusion	Diversity increased over time in all stress treatments: herbivore exclusion, nutrient enrichment and herbivore exclusion * nutrient enrichment	+,+,+	Jessen *et al.* 2013
Overfishing	Macroalgae Contact	Richness increased next to all algae, and Chao1 was significantly higher in all treatments (i.e. corals touching *Dictyota menstrualis*, *Galaxuara*	+,+,+,+,+	Vega-Thurber *et al.* 2012

		obtusata, *Halimeda tuna*, *Lobophora variegate*, *Sargassum polyceratium*)		
		Diversity increased next to CCA and *Dictyota bartayresiana*; Decreased next to *Halimeda opuntia* and turf algae	++--	Barott *et al.* 2012
Overfishing Pollution, Climate Change	Thermal Stress, Nutrients, Macroalgae Contact	Richness increased next to algae	+	Zaneveld *et al.* 2016

Figures

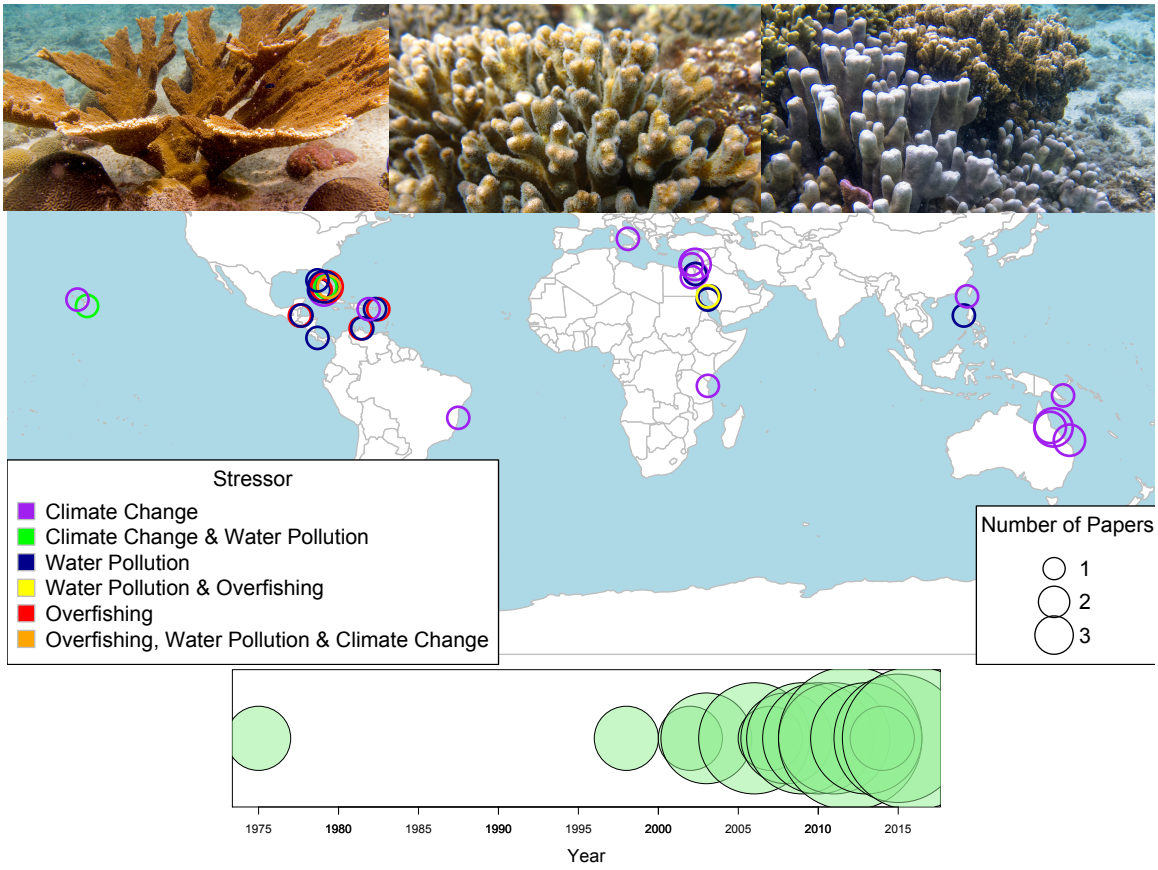

Figure 2.1. A world map of all studies evaluating the impact of stressors on the microbiome, climate change (purple), climate change and water pollution (green), water pollution (blue), water pollution and overfishing (yellow), overfishing (red), and overfishing, water pollution and climate change (orange). The size of the bubble refers to the number of papers at that latitude/longitude. Pictures on the top correspond to coral species included in these papers (left to right: *Acropora palmata* (photo by Ryan McMinds), *Pocillopora damicornis* (photo by Joseph Pollock), and *Porites cylindrica* (photo by Ryan McMinds)).

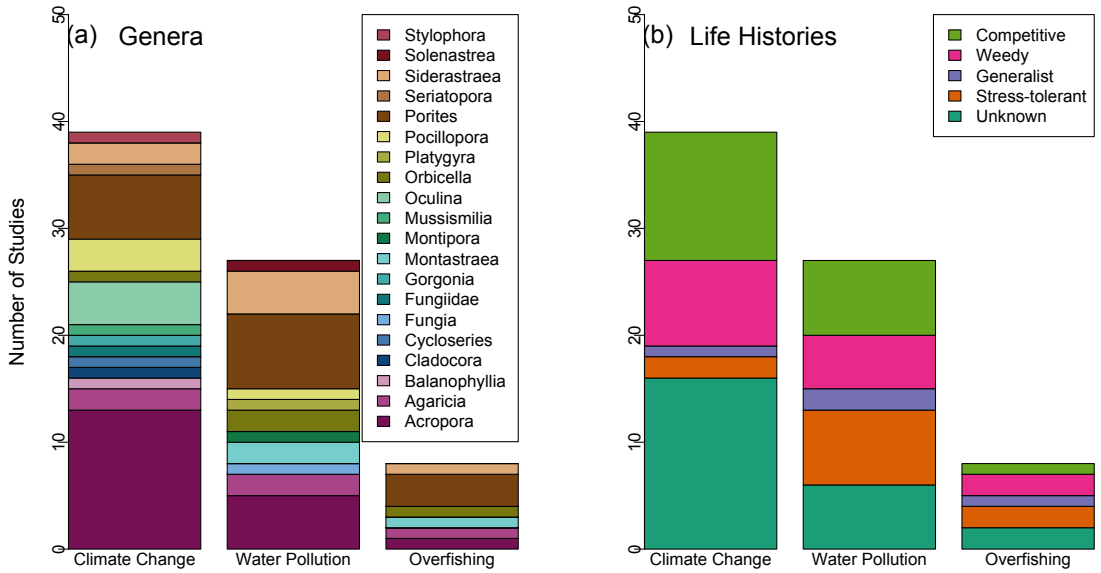

Figure 2.2. Plot of a) coral genera and b) coral life-histories included in all studies on the impact of climate change, water pollution and overfishing on the reef microbiome (from Table 1). Coral life-histories are taken from (Darling *et al.* 2012).

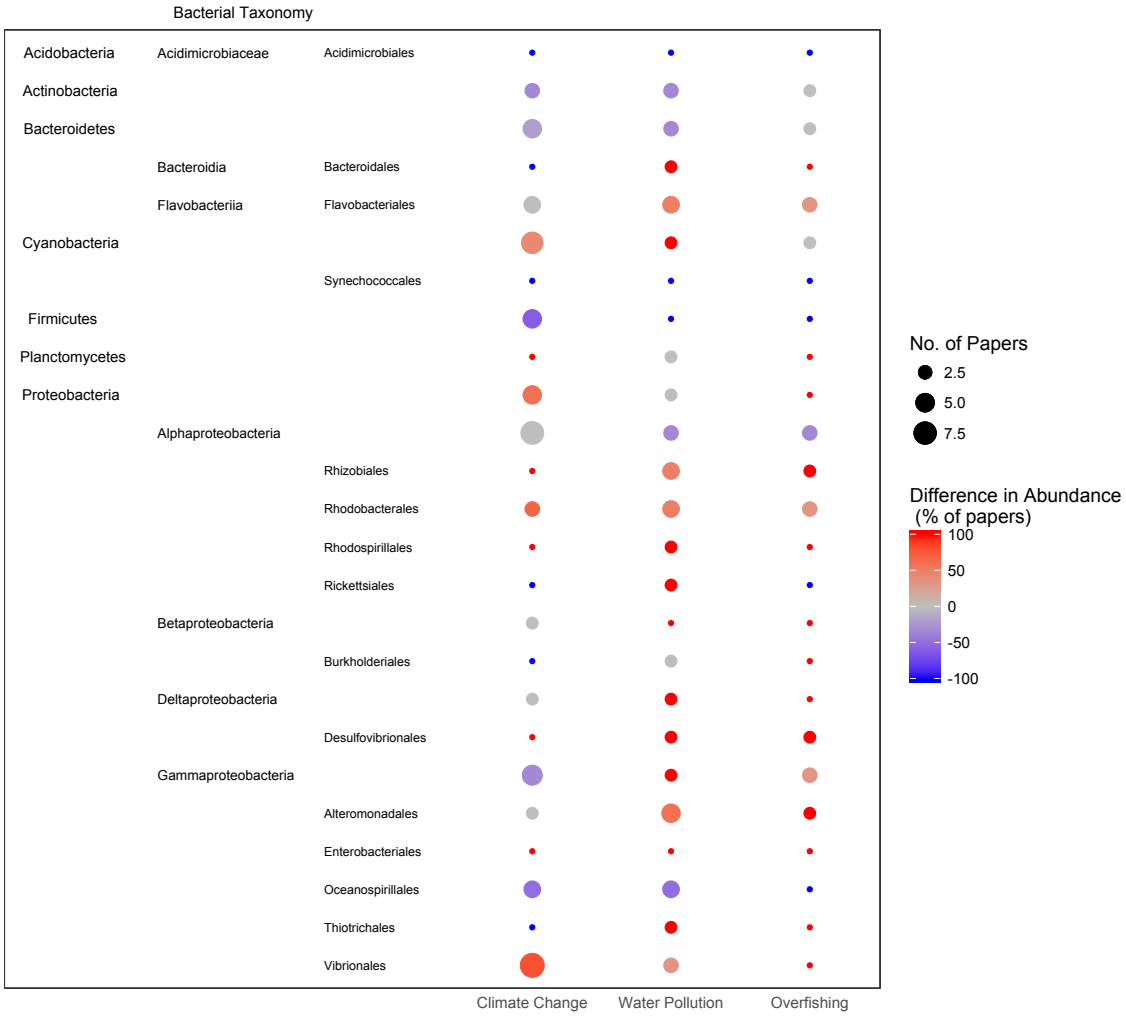

Figure 2.3. Summary of the number of papers (indicated by bubble size) showing differences in bacterial taxa (red=taxa overrepresented, blue=taxa underrepresented) during stress events (i.e. climate change, water pollution or overfishing).

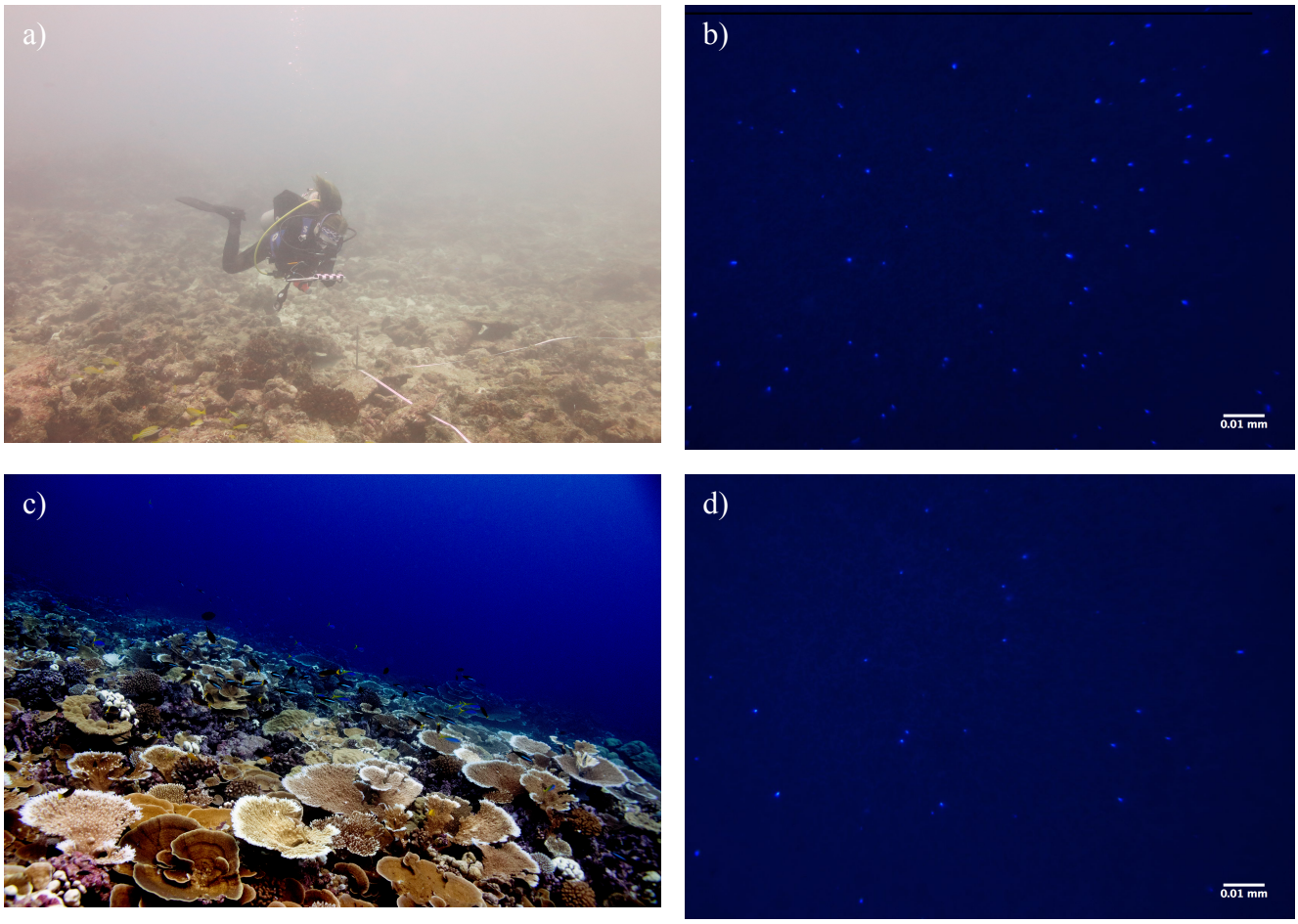

Figure 2.4. Photos demonstrating a) a highly disturbed reef (photo by John Burns) and b) the bacteria in the water column of the highly disturbed reef and c) a near-pristine reef (photo by Kristina Tietjen) and d) the bacteria in the water column of a near-pristine reef versus both found on Kiritimati (Republic of Kiribati) filtered with 2mls of water, stained with DAPI and photographed under an epifluorescent microscope.

Chapter 3- Impacts of human disturbance and a bleaching hotspot on the coral microbiome

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3.1 Introduction

The study of how microbial communities contribute to the macro ecological world is rapidly advancing due to the discovery of widespread microbial abundance and diversity, and the decreasing cost of next-generation sequencing. Host associated microbes, generally termed the "microbiome", are especially important in host ecology as they can provide protection (Stabb and Visick 2013; Welsh *et al.* 2016), nutrition (Savage 1986; Lesser *et al.* 2004) or cause disease (Tompkins *et al.* 2015). With their rapid turnover and short life span, microbiomes are especially important components of host responses to stressors and assisting host adaptation (Bosch and McFall-Ngai 2011; McFall-Ngai *et al.* 2013).

The extensive diversity and abundance of the coral microbiome was discovered in 2002 (Rohwer *et al.* 2002) with corals harbouring distinct communities from the surrounding water and sediment (McKew *et al.* 2012) and unique communities within different coral compartments (i.e. coral tissue, skeleton, mucus, gut) (Sweet *et al.* 2010; Blackall *et al.* 2015). As most of these coral-associated microbes remain unculturable, the hypothesized roles so far range from nitrogen acquisition (Lesser *et al.* 2004; Lema *et al.* 2012; Ceh *et al.* 2013), sulfur cycling (Wegley *et al.* 2007; Raina *et al.* 2009), and protection (Ritchie 2006; Rypien *et al.* 2010; Kvennefors *et al.* 2011; Krediet *et al.* 2012; Welsh *et al.* 2016). However, some of these microbes can harm the coral by causing bleaching (Kushmaro *et al.* 1998; 2001; Ben-Haim and Rosenberg 2002) or disease (Sutherland *et al.* 2011). Thus, the stability and composition of these associated microbial communities is essential to coral and overall reef health.

The coral microbiome likely plays an important role in coral resistance to and resilience from (Ainsworth and Gates 2016) stressors currently threatening reefs

worldwide (Gardner 2003; De'ath *et al.* 2012; Ainsworth *et al.* 2016). However, stress events on coral reefs induce changes in the community composition of the coral microbiome. For example, during thermal stress events the microbial community can shift towards pathogenic and virulence genes potentially harmful to the coral (Bourne *et al.* 2007; Littman *et al.* 2011; Tout *et al.* 2015). Local stressors, such as water pollution, can also induce changes in the microbiome by directly adding pathogens/opportunists and increasing diversity (Sutherland *et al.* 2010; Morrow *et al.* 2012; Ziegler *et al.* 2016). Furthermore, local and global stressors commonly interact, further disrupting the coral microbiome (Zaneveld *et al.* 2016). Disruptions of the coral microbiome can be harmful to coral health and cause bleaching or even tissue death (Glasl *et al.* 2016). However, the combined influence of human disturbance and thermal stress on coral-associated microbial communities remains unclear.

Here, we examine how the coral-associated microbial community varies among individual coral colonies, and how this relationship is influenced by human disturbance and thermal stress from a bleaching hotspot. Using 103 coral samples from two coral species (i.e. *Porites lobata* and *Montipora foliosa*) from four sites at Kiritimati Atoll, Kiribati, we ask how human disturbance and thermal stress influence microbial taxa and community structure within the coral microbiome?

3.2 Materials & Methods

3.2.1 Sample Collection

Samples were collected along four fringing reef sites at Kiritimati Atoll (1.8721˚ N, 157.4278˚ W, Kiribati) at ~ 10 m depth (Figure 3.1). Two sites are considered to be in a high disturbance category as they face high fishing pressure (Walsh 2011) and runoff

due to their close proximity to the large villages of Tabwakea (2311 people) and London (1879 people) (Kiribati National Statistics Office 2012). The other two sites are considered low disturbance, as they are not near any villages and face low fishing pressure (Walsh 2011). Transects were laid out to 60m horizontally to the land when possible. A dive team tagged corals using zipties and z-spared aluminum numbered tags to monitor the same coral over multiple field seasons. Samples were collected from April 30-May 10 and from July 2-19, 2015.

Temperature data was recorded using Sea-Bird Temperature Loggers installed on steel stakes at each site, except for one site at the low disturbance level. The temperature loggers recorded data at 1-hour intervals. Surface water quality samples were collected at one-meter depth at each site, and immediately stored in EPA approved vials for nutrient analysis. These nutrient samples were taken within a three-day window of the collected corals. One liter of water was collected approximately ~1m above the benthos along the transect with collapsible containers. Five milliliters of water was syringed out and fixed to a final concentration of 2% formaldehyde for microbial counts while the remaining sample was filtered on a 0.2um filter paper within four hours of collection and placed in RNAlater for sample preservation for 16S sequencing. There were three-four microbial count water samples per site (i.e. 31 samples total across both field seasons) and three-four 16S water samples per site except for the high disturbance level in the pre-bleaching hotspot due to sampling error (i.e. 23 samples total). Coral benthic surveys were conducted along a 60m transect with 1m X 1m photo quadrats (n=15-30 per site) to evaluate benthic percent cover (i.e. algae, CCA, and healthy coral cover).

111 coral samples were collected along the 60m transect (the same as the benthic surveys) during both sampling expeditions (i.e. pre-bleaching and bleaching hotspot). Coral colonies were tagged previously in August 2014 for long-term monitoring, and new colonies were tagged to increase the sample size due to lost colonies. *Porites lobata* was sampled with a chisel to scrape off coral tissue while *Montipora foliosa* was sampled by breaking off an edge piece. The coral samples were then put on ice when reaching the surface, and processed the same evening by placing them in centrifuge tubes and avoiding most of the coral skeleton.

Coral samples were placed on ice in a cooler and then processed by the end of the day and stored in a -20° C deep freeze during the rest of the expedition. The corals and water samples were immediately stored in the -20° C freezer while water and sediment samples fixed in 2% formaldehyde were placed in the fridge overnight and put in the -20° C the next night. Samples were then stored at -80° C for long-term storage at the University of Victoria.

3.2.2 Environmental Sample Processing

To determine the number of degree heating weeks using our temperature data, we used the equation by NOAA coral reef watch (Liu et al. 2006):

(1) $DHWs = 0.5 * summation\ of\ previous\ 24\ twice - weekly\ hotspots$

with a hotspot defined as:

(2) $Hotspot = SST - MMM$

A "bleaching hotspot" is defined when the sea surface temperatures exceed the MMM (maximum monthly mean) by 1° C, implying that SSTs are going to cause coral bleaching. We defined the MMM (28.1366° C) from NOAA data on the Northern Line

Islands (NOAA Coral Reef Watch 2013). Therefore our sampling time points were designated as: April 30-May 10="pre-bleaching hotspot" and July 2-19, 2015="bleaching hotspot" (Figure 3.2).

The three technical replicates of surface water per site were processed for nitrate plus nitrite and phosphate concentrations at the Institute of Ocean Sciences (IOS). The 2% formaldehyde reef depth water samples were filtered (1-2mls depending on the visibility) on a 0.2um black polycarbonate filter paper. ¼ of the filter paper was placed on a slide and stained with DAPI. Using a Zeiss Universal epifluorescent microscope, 20-31 photos were taken per slide using a grid search pattern to ensure no two fields of view overlapped. An average of 30.82 bacteria were counted per field of view to ensure no statistical bias from counting (Kirchman *et al.* 1982). Coral benthic percent cover for each site, except for one site in the low disturbance level in the pre-bleaching time point, was computed from the benthic photos using the program Coral Net, selecting 100 random points in the quadrat and identifying the benthos.

3.2.3 DNA Extraction

All DNA extractions (i.e. reef water and coral for each field season) were performed in random order. The DNA was extracted following the Earth Microbiome Protocol (with incubation in a hot water bath of 65°C for 10 minutes and elution period of 10 minutes) using Mobio Powersoil DNA Isolation kits (EMP 2016). For each DNA extraction, we used approximately 50ul of coral tissue and ¼ of a filter paper (i.e. the filtered water sample).

3.2.4 PCR Amplification and Sequencing

The 16S rRNA V4 region was amplified using modified Earth Microbiome Project primers: 515fb/806rb (515fb- GTGYCAGCMGCCGCGGTAA, 806rb-GGACTACNVGGGTWTCTAAT) following a two-step PCR protocol. To prevent contamination, PCR tubes and nuclease free water were placed under ultraviolet light for ten minutes prior to use, and the first PCR was prepared in a PCR fume hood. The first PCR was a triplicate 15ul reaction (total of 45ul) with 0.5ul of template DNA and 14.5ul of a master mix (i.e. 6ul nuclease-free water, 7.5ul AccuStart II PCR ToughMix polymerase (Gaithersburg, MD), 0.5ul forward primer and 0.5ul reverse primer). PCR conditions followed EMP thermocycler conditions of 1) 94°C for 3 minutes, 2) 94°C for 45 seconds, 3) 50°C for 60 seconds, 4) 72°C for 90 seconds, 5) repeating steps 2-4 35 times, 6) 72°C 10 minutes, 7) 4°C hold. The pooled triplicates were then run on a 2% agarose gel with 1ul-3ul of DNA template. The target bands (i.e. the 16S bacterial bands) were then cut under UV light to avoid the coral mitochondrial band as coral mitochondria is also amplified from 16S primers. The gel slice was then cleaned using Promega Wizard Gel Clean Up. For the second PCR, the water was exposed to ultraviolet light for ten minutes prior to the PCR and consisted of 5-10ul of template DNA, 12.5 AccuStart II PCR ToughMix polymerase, 1ul forward schloss barcodes (i.e. sequencing adaptors) and 1ul of reverse schloss barcodes, and topping up with nuclease free water for a 25ul reaction. The second PCR consisted of only 12 cycles instead of 35 with the same thermocycler conditions to allow the barcodes to attach. The product of the second PCR was then visualized on a 1% agarose gel to ensure the fragment had amplified. 22.5ul of this product was then cleaned with an Agencourt AMPure PCR Bead Cleanup. The genomic high-sensitivity double stranded DNA concentration was then measured using a

qbit fluorometer and samples were pooled with different volumes to ensure samples were equimolar ratio for sequencing. Negative control samples (i.e. 0.02 um filtered and ultraviolet exposed water) were also included through these steps from the first PCR through to the equal molar pooling. Samples were then sequenced using 2X300bp reads on the MiSeq Illumina platform at Oregon State University's Center for Genome Research and Biocomputing Core Laboratories.

3.2.5 Sequence Analysis

The sequence data were processed using Quantitative Insights into Microbial Ecology (QIIME) (version 1.9.1) (Caporaso *et al.* 2010) for demultiplexing, quality filtering and clustering. After trimming the primers, paired reads were joined to increase the length of reads, check the overlapping sequences, and increase the quality. The maximum allowed percent difference between regions was 20% and the minimum allowed overlap required in the base pairs to join the pairs was 20 for the read to be kept. The primers were then trimmed off of forward reads that could not be joined with their reverse read, and these forward reads were kept along with the successfully joined paired reads. Quality filtering during split_libaries_fastq included the parameters: p=0.75 (minimum number of consecutive high quality base calls), q=20 (maximum unacceptable phred quality score), r=3 (maximum number of low quality base calls) following QIIME suggestions (although note the higher q score). Singletons were automatically filtered out during OTU picking using the GreenGenes (13_8) database (McDonald *et al.* 2011) and chimeras were filtered out using "Usearch61" (Edgar 2010). OTUs were determined using open reference OTU picking where samples are first compared against a reference database (i.e. closed reference OTU picking) and then reads that did not match the

reference database were clustered together using de novo OTU picking (i.e. all at 97%). Bokulich *et al.* (2013) suggest filtering out OTUs not found at 0.005%, which they argue are spurious reads (i.e. errors). However, their suggestion was for forward reads and our data consists of paired-end reads (i.e. higher quality). We therefore did not filter our OTUs at 0.005% as these low abundance OTUs are high quality and may represent the rare biosphere that are important within corals. We also increased the phred quality score from 3 to 20. Sequences identified as mitochondria and chloroplasts were filtered out.

As coral samples are low in biomass, and their mitochondria amplify with 16S primers, the samples were compared to the six negative controls that were sequenced in the same run (see Appendix A). Taxa identified within the *Montipora foliosa* samples were surprisingly similar to the negative controls so we filtered out the top 10 OTUs in the negative controls from the coral and water samples. These top 10 OTUs comprised ~79% of all the negative controls so these taxa would have the largest impact on the analysis. We recognize that we may be losing some diversity due to this filtering, but suggest that with 16S primers, one can never capture all of the diversity, and it is therefore better to be conservative.

3.2.6 Statistical Analyses

For the environmental data, we counted the number of bacterial cells per ml for each water sample and compared average abundance between samples collected for different disturbance levels and expeditions. We used linear models to assess the major drivers of bacterial abundance in a global model with sampling time point (i.e. pre-bleaching or bleaching) and disturbance level (i.e. high or low) as interacting explanatory covariates. Site was nested as a fixed effect in human disturbance. We included site as a

fixed nested effect within human disturbance, as it did not contain enough levels (i.e. four) to be included as a random effect (Zuur *et al.* 2009). Including site as a nested fixed effect can account for non-independence between sites when there are not enough levels to treat it as a random effect (Schielzeth and Nakagawa 2013). We used the package lsmeans in R to get least squares means for the best model (Lenth 2016), but could not include site nested within human disturbance. Therefore, we plotted the least squares means per site to demonstrate site similarity and then conducted post-hoc contrasts to determine significant differences between disturbance levels. Since these contrasts do not consider the impact of site variation, plots of coefficient estimates were used to examine differences across sites.

To deal with differences in library size (i.e. number of reads per sample, sampling effort) between samples, we transformed our data into relative abundances for the multivariate analyses. Rarefying samples (i.e. randomly subsampling to the small acceptable read number) is common practice in 16S rRNA studies, however McMurdie and Holmes (2014) demonstrated this procedure is unacceptable for differential abundance and clustering statistics as it throws away data, increases the chance of Type I error and adds a step of randomness. McMurdie and Holmes (2014) demonstrate that for clustering methods with the Bray-Curtis distance, converting read abundance to proportions outperforms other variance stabilizing techniques (particularly rarefying). It is also becoming more common to use these proportions in microbiome studies (Hester *et al.* 2016; Lagkouvardos *et al.* 2017).

We performed our statistical analyses following recommendations by Anderson and Willis (2003): 1) robust unconstrained ordination, 2) constrained ordination testing a

hypothesis, 3) rigorous statistical test of this same hypothesis, and 4) determining species responsible for these patterns (albeit we did not statistically evaluate this). We used both an unconstrained (principal coordinates analysis (PCoA)) and constrained (canonical analysis of principal components (CAP)) with the Bray-Curtis coefficient. For the constrained CAP, we conducted a backwards-stepwise ANOVA with 9999 permutations to determine the best model starting with the terms: coral species, human disturbance and bleaching, as well as expedition when comparing the pre-bleaching to bleaching hotspot.

Next, we evaluated differences in microbial communities using a PERMANOVA with 9999 permutations. For the pre-bleaching and bleaching hotspot analyses conducted separately, we conducted both a CAP and PERMANOVA with the starting factors: coral species, human disturbance (with site nested) and bleaching. For all the data, pre-bleaching and bleaching hotspot combined, we conducted the analyses CAP and PERMANOVA with the starting factors coral species, human disturbance (with site nested), bleaching and expedition (i.e. pre-bleaching and bleaching hotspot). To account for repeated measures of corals sampled in both the pre-bleaching and bleaching hotspot, we included "coral colony id" as a random effect. For the reef water samples, we conducted a PERMANOVA and CAP with the fixed effect human disturbance with site nested.

Additionally we evaluated differences in beta diversity by using the relative abundance transformed OTU table. To evaluate differences in beta diversity (i.e. distance to centroid), we used a PERMADISP test with 9999 permutations on each coral species separately. We conducted this test between human disturbance levels in both the pre-

bleaching and then the bleaching hotspot. In addition, we evaluated changes in beta diversity between the pre-bleaching and bleaching hotspot within each disturbance level.

Finally, we evaluated differences in alpha diversity for the pre-bleaching hotspot, the bleaching hotspot, and the combined pre-bleaching and bleaching hotspot. For alpha diversity analyses, we rarefied our OTU table to the lowest read number to deal with differences in library sizes (i.e. not using the relative abundance OTU table). We utilized linear models to evaluate changes in the Shannon index, as this index is useful in situations where rare species are expected to be as important as abundant ones (Morris *et al.* 2014).

(3) $Shannon = -\sum p_i ln p_i$

In the Shannon equation p_i is the proportion of individuals in species i. For the pre-bleaching and the bleaching hotspot (evaluated separately) we used model selection (i.e. identifying the model with the lowest AICc) to determine the best model (Burnham and Anderson 2002), with coral species and human disturbance as interacting explanatory covariates, and site nested within human disturbance. We used linear mixed effects models in the package lme4 (Bates et al. 2015) when evaluating pre-bleaching and the bleaching hotspot together and included coral colony id as a random effect. To account for species-specific differences, we conducted our analyses on *Montipora foliosa* and *Porites lobata* separately in the combined pre-bleaching and bleaching analysis. We extracted the least squares means for the best model using the package lsmeans. All of the statistical analyses were conducted in R using the phyloseq (McMurdie and Holmes 2013), vegan (Oksanen *et al.* 2016), lsmeans (Lenth 2016) and MuMIn (Barto'n 2016) packages.

3.3 Results

The unfiltered OTU table for both coral and water samples consisted of 2,915,759 total reads, with a median of 13,194 reads per sample. However, once mitochondria and chloroplasts were filtered out, there were 2,304,059 total reads and a median of 9,563 reads per sample. After filtering out the top 10 OTUs within the negative controls, there were 2,104,240 reads and a mean of 8,176 reads per sample. We discarded eight coral samples as they had less than 867 reads. After filtering, we retained 103 coral and 23 water samples. There were 9,910 distinct OTUs within the coral samples and 12,232 distinct OTUs within the water samples.

3.3.1 Human Disturbance and a Bleaching Hotspot Drive Differences in Environmental Data

In the pre-bleaching hotspot, the average temperature was 28.87° C compared to 29.43° C in the bleaching hotspot (Figure 3.3). The bleaching hotspot at Kiritimati began on May 24, 2015 and Kiritimati experienced 12.86 DHWs by the end of our sampling period in July (Figure 3.4). The low disturbance sites had higher nitrate plus nitrite (uM) (Pre-bleaching Hotspot: low=2.78, high=1.87; Bleaching Hotspot: low=3.83, high=0.95) and phosphate (uM) (Pre-bleaching Hotspot: low=0.52, high=0.49; Bleaching Hotspot: low=0.61, high=0.39) during both sampling expeditions (Figure 3.5). There was no significant difference in bacterial abundance between sampling expeditions. The best model included expedition and human disturbance (with site nested) (AICc: 767.29, next AICc: 768.63 (human disturbance/site only)). During both sampling time points, the high disturbance level had significantly higher microbial cells per ml than the low disturbance level (Figure 3.6, z-ratio=12.54). In addition, the water microbial communities between disturbance levels were significantly different (Figure 3.7, PERMANOVA, F=13.94, p-

value=0.0001) (CAP, 77.3% of the variation explained). Finally, the low disturbance level had lower algal cover (30.6%), higher healthy coral cover (55.1%) and higher crustose coralline algal cover (6.9%) than the high disturbance level (Algae: 36.5%, Healthy Coral: 2.3%, CCA: 0.06%) (Figure 3.8).

3.3.2 Human Disturbance and a Bleaching Hotspot Drive Differences in Coral Microbial Community Composition

Coral species, human disturbance and sampling time point (i.e. pre-bleaching to bleaching hotspot) all contributed significantly to variation in the coral microbiome. Utilizing an unconstrained ordination that incorporates all the variation within the data, there is a visual separation between coral species, disturbance levels and expedition (i.e. pre-bleaching and bleaching hotspot) (Figure 3.9a, 3.10a, 3.11a). During the pre-bleaching hotspot, *M. foliosa* and *P. lobata* had significantly different microbial communities between each other (PERMANOVA, F= 7.18, p=0.0001) and between disturbance levels (PERMANOVA, F=3.65, p=0.0007) (CAP Best Model: coral species + human disturbance/site, 27.55% of the variation explained) (Figure 3.9b). When evaluating the bleaching hotspot alone, *M. foliosa* and *P. lobata* had significantly different communities between each other (PERMANOVA, F=7.19, p=0.0001) and disturbance levels (PERMANOVA, F=2.41, p=0.0013) (CAP Best Model: coral species + disturbance/site, 18.48% of the variation explained) (Figure 3.10b). When comparing the pre-bleaching to the bleaching hotspot, the PERMANOVA results and CAP best model did not agree. The PERMANOVA found *M. foliosa* and *P. lobata* had significantly different communities from each other (F=12.45, p=0.014), between expeditions (i.e. the pre-bleaching and bleaching hotspot) (F=1.91, p=0.0091), and

between human disturbance levels (F=3.22, p=0.0140). However, the CAP best model only included expedition while controlling for local colony (Figure 3.11b, CAP: 1.7% of the variation). Therefore, with such low variation explained by the CAP model, we decided the PERMANOVA had more robust results.

3.3.3 Responses of Beta Diversity to Human Disturbance and a Bleaching Hotspot

We also detected species-specific responses of beta diversity between disturbance levels and sampling time point (i.e. pre-bleaching and bleaching hotspot). Within the pre-bleaching hotspot, *P. lobata* had significantly higher beta diversity (i.e. variation) in the high than low disturbance level (F =24.72, p =0.0001) whereas *M. foliosa*'s beta diversity was not significantly different between disturbance levels (Figure 3.12). However, during the bleaching hotspot, there was no significant difference in beta diversity between disturbance levels for both *P. lobata* and *M. foliosa* (Figure 3.13). *P. lobata* and *M. foliosa* each had significantly higher beta diversity at the bleaching hotspot than the pre-bleaching hotspot within the low disturbance level (F=35.19, p=0.0001; F=4.45, p=0.0478). However, there was no difference in beta diversity between the pre-bleaching and bleaching hotspot within the high disturbance level (Figure 3.14).

3.3.4 Responses of Alpha Diversity to Human Disturbance and a Bleaching Hotspot

Additionally, we found differences in alpha diversity between coral species, disturbance levels and sampling time point (i.e. pre-bleaching and bleaching hotspot). During the pre-bleaching hotspot, the best model for alpha diversity included coral species and human disturbance (AICc=109.56, next AICc=113.09 (coral species * human disturbance/site). Both *P. lobata* and *M. foliosa* had significantly higher alpha diversity at the high versus the low disturbance level (t-ratio=4.319, p-value=0.0001), while *P. lobata*

had significantly lower than *M. foliosa* at both disturbance levels (Figure 3.15, t-ratio= 3.716, p-value=0.0007). The best model to fit the bleaching hotspot, describing alpha diversity, included just coral species (AICc=197.41, next AICc=201.76 (coral species + human disturbance). During the bleaching hotspot, *M. foliosa* had significantly higher alpha diversity than *P. lobata* (t-ratio=4.14, p-value=0.0001) (Figure 3.16). When including all the data (i.e. coral samples from the pre-bleaching and the bleaching hotspot) the best model for microbial alpha diversity in both *P. lobata* and *M. foliosa* only included human disturbance (with site nested) (*P. lobata*: AICc=175.39, next model AICc=177.19 (expedition); *M. foliosa*: AICc=141.01, next AICc=142.34 (expedition)). *P. lobata* had significantly higher alpha diversity in the high than the low disturbance level (z-ratio=2.884, p-value=0.0039), while *M. foliosa* almost had significantly higher alpha diversity within the high compared to the low disturbance level (z-ratio=1.86, p-value=0.0625) (Figure 3.17).

3.3.5 Differences in Coral Microbial Community Composition Between Disturbance Levels and a Bleaching Hotspot

Coral microbiome composition varied between coral species, human disturbance levels and the pre-bleaching and bleaching hotspot. During the pre-bleaching hotspot, Proteobacteria dominated *P. lobata* at both disturbance levels (mean relative abundance= 99% (L) and 77% (H). *P. lobata*, especially at the low disturbance, was dominated by Vibrionales (87% (L), 28% (H)) with the families, Pseudoaltermonadaceae and Vibrionaceae at 49% and 38% relative abundance respectively for the low disturbance site and 11% and 17% respectively at the high disturbance site. The family, Endozoicimonaceae was found at 10% and 9% relative abundance for the low and high

disturbance level, respectively. Rhodobacteraceae was at 10% relative abundance in the high disturbance level (see Appendix B). *M. foliosa* was mainly composed of Proteobacteria (72% (L) and 51% (H)) and Firmicute*s* (13% (L) and 10% (H)). Rhodobacterales were found at 13% relative abundance for both low and high disturbance levels, while Burkholderiales and Rhizobiales comprised of 13% and 9% in the low disturbance level. Rhodobacteraceae comprised of 13% and 12% relative abundance for high and low disturbance levels (see Appendix B).

During the bleaching hotspot, Proteobacteria dominated *P. lobata* samples at 86% and 78% relative abundance in the low and high disturbance levels. Vibrionales was the most abundant order in both the high and low disturbance level at 56% and 43% relative abundance. Additionally, Oceanospirillales were at 25% relative abundance in the low disturbance site. Pseudoalteromonadaceae and Vibrionaceae were dominant at the high (29%, 27%) and low (19%, 25%) disturbance sites. Additionally, Endozoicimonaceae was at 24% relative abundance at the low disturbance level. For *M. foliosa*, Proteobacteria dominated the high and low disturbance levels at 68% and 65% relative abundance. At the order level, Oceanospirillales were at the highest relative abundance at 14% and 12% for high and low disturbance levels. In the low disturbance level, Myxococcales were at 12% relative abundance, while Rhodobacterales, Vibrionales and Alteromonadales were at 8%, 8% and 7% relative abundance in the high disturbance level. For microbial families, Endozoicimonaceae, Rhodobacteraceae and Vibrionaceae were at 12%, 8% and 7% relative abundance at the high disturbance level while Oceanospirillaceae, Rhodobacteraceae, Pseudomonadaceae and Alcanivoraceae were all at 4-5% relative abundance in the low disturbance level (see Appendix B).

3.4 Discussion

Overall, coral microbiome composition was driven by coral host species, human disturbance and thermal stress from a bleaching hotspot. Human disturbance and thermal stress each induced changes in the abundance of potential symbionts (e.g. *Pseudoaltermonas*, *Endozoicomonas*), opportunists (e.g. Rhodobacterales) and pathogens (e.g. Vibrionaceae). Human disturbance induced increases in beta and alpha diversity, while thermal stress increased beta diversity only within the low disturbance level and homogenized alpha diversity between disturbance levels. Microbiome differences between coral species and human disturbance lessened during the bleaching hotspot as both became homogenized after nearly 13 DHWs of thermal stress.

3.4.1 Human Disturbance Increases Alpha and Beta Diversity Within the Microbiome

Human disturbance also influenced the coral microbiome by increasing microbial alpha diversity in both coral species. Coral microbiome diversity has been shown to increase under increasing seawater temperatures (Lee *et al.* 2016), ocean acidification (Meron *et al.* 2011), water pollution (Ziegler *et al.* 2016) and within diseased corals (Sunagawa *et al.* 2009), suggesting that increased microbial diversity indicates a stressed coral. Thus, the corals within the high disturbance site are likely stressed and experiencing invasions from opportunistic and potentially pathogenic bacteria, consequently increasing microbial alpha diversity. This may be due to the higher amount of bacteria in the water column at the high disturbance level.

In addition, *P. lobata* had significantly higher beta diversity (i.e. variation from coral to coral) in the high disturbance level than the low disturbance level. Increased variation implies a stressed microbiome and is suggested to demonstrate the hosts

decrease in regulation of its microbiome for both chimpanzees (Moeller *et al.* 2013) and corals (i.e. specifically from temperature extremes and algal contact) (Zaneveld *et al.* 2016). This increase in variation may be due to the coral immune pathway being suppressed by coral pathogens (e.g. *Vibrio coralliilyticus* (Vidal-Dupiol *et al.* 2014)) thus increasing microbiome variation among colonies. Thermal stress also suppressed the coral immune pathway (Vidal-Dupiol *et al.* 2014), so stress from human disturbance may play similar role, although this has not been tested empirically. Therefore, increased variation within the high disturbance level suggests that the corals are stressed and cannot fully regulate their microbiome. This is supported by the higher abundance of *Pseudoaltermonas* in *P. lobata* at the low disturbance level. *Pseudoaltermonas* has been shown to produce antimicrobial compounds that protect the coral host from invasions (Shnit-Orland *et al.* 2012). Thus, microbes within this genus may be protecting *P. lobata* from invasions within the low disturbance level and keeping beta diversity low.

3.4.2 A Bleaching Hotspot Results in Microbiome Homogenization Between Disturbance Levels

During the bleaching hotspot, there was no longer a significant difference in alpha or beta diversity between human disturbance levels. Bleaching hotspots may serve as the primary determinant of coral microbiome alpha and beta diversity, thus during the bleaching hotspot, variation in human disturbance is not as important. In support of this, Lee *et al.* (2012) found that corals clustered more by environmental gradients than by coral species and within more pristine sites, there was more separation between coral species.

Furthermore, there were no significant difference in alpha diversity between sampling time points, suggesting alpha diversity did not increase under stress as we would expect (Bourne *et al.* 2007; Santos *et al.* 2014; Lee *et al.* 2016). This may be due to the corals already experiencing increased temperatures during the pre-bleaching hotspot and without prior expeditions; we cannot know the underlying diversity values. However, there was a significant increase in microbial beta diversity from the pre-bleaching to bleaching time point for both *P. lobata* and *M. foliosa* in the low disturbance level. Yet there was no change in beta diversity for corals in the high disturbance level. This suggests that corals in the low disturbance level were able to regulate their communities prior (e.g. *Pocillopora damicornis* immune pathways were activated in the presence of non-virulent bacteria to potentially perform bacterial regulation (Vidal-Dupiol *et al.* 2014)) to this intense thermal stress event. However, the bleaching hotspot destabilized the microbiomes and led to greater variation among coral colonies, as shown previously by Zaneveld *et al.* (2016). This increase in microbiome variation may be due to the thermal stress or increase in pathogens inducing a decrease in coral immunity gene expression (Vidal-Dupiol *et al.* 2014) and thus less regulation by the coral of microbiome members.

3.4.3 Microbiome Composition is Driven by Human Disturbance

Microbial communities were significantly different between disturbance levels, however there was more distinct clustering (as demonstrated by our CAP analysis) by coral host species in the low than the high disturbance level. Lee *et al.* (2012) also found this trend, with more separation between coral species in a pristine site than more impacted site, suggesting corals are more selective of their microbiome in pristine reefs.

This consistent microbial community in the low disturbance level, dominated by Vibrionales, specifically the families Pseudoaltermonadaceae and Vibrionaceae, suggests that all the corals were responding in the same way to elevated temperatures. Additionally, microbes within the genus *Pseudoaltermonas* and family Pseudoaltermonadaceae, were more abundant in the low disturbance level. *Pseudoaltermonas* is thought to be a coral symbiont, and has been shown to inhibit the growth of the coral pathogen, *Vibrio shiloi* (Shnit-Orland *et al.* 2012). Thus, *Pseudoaltermonas* and related genera within Pseudoaltermonadaceae could be playing a protective role in the low disturbance site. Additionally, increases in the presence of Vibrionales prior to extreme thermal stress could be a pre-indicator of bleaching, as was previously suggested (Bourne *et al.* 2007). The corals in low disturbance could be responding to the increase in temperatures before the high disturbance level, because the low disturbance level had greater visibility (i.e. > 100 ft. versus ~ 30ft). Thus, the greater visibility could correlate with increased light stress which can exacerbate coral bleaching (Abrego *et al.* 2008). Additionally, the temperatures during the pre-bleaching hotspot may be hot enough to induce pathogenicity as they were greater than the local MMM (Maynard *et al.* 2015; Zaneveld *et al.* 2016).

Microbial communities in *M. foliosa* differed significantly between disturbance levels, however this difference was less pronounced than in *P. lobata*. Both Burkholderiales and Rhizobiales were more abundant in the low disturbance level; these are potential nitrogen fixers within corals that are thought to be important members of the coral holobiont (Lema *et al.* 2012; Morrow *et al.* 2012; Ceh *et al.* 2013; Lema *et al.* 2014).

3.4.4 A Bleaching Hotspot Drives Microbiome Composition More Than Human Disturbance

During the bleaching hotspot, coral associated microbial communities were different between coral species and disturbance levels. However, these two factors explained less of the variation compared to the pre-bleaching hotspot (species: 18.4% to 10.5%, human disturbance: 5.1% to 3.8%), suggesting the corals between disturbance levels and species were becoming more similar. Anthropogenic impact (Ziegler *et al.* 2016) and disease (Frias-Lopez *et al.* 2004; Roder *et al.* 2014) have been shown to decrease coral host specificity and increase coral microbiome similarity, likely due to coral species responding to a stressful event in similar ways. Therefore, corals are potentially regulating their community less to local conditions and microbial communities are responding similarly to the thermal stress between human disturbance levels and coral species. As stated previously, Lee *et al.* (2012) found that corals clustered more by environmental gradients than by coral species and there was more separation between coral species within the pristine sites. Therefore, during this stress event on Kiritimati, the increased temperatures may drive microbiome composition more than human disturbance.

Although *P. lobata's* and *M. foliosa's* microbiomes were still significantly different in the bleaching hotspot, the difference was less pronounced than in the pre-bleaching hotspot. Once again, *P. lobata* was dominated by Vibrionales at both disturbance levels, likely due to pathogenesis (Tout *et al.* 2015) and lower amounts of *Pseudoaltermonas* to ward off invaders (Shnit-Orland *et al.* 2012). Additionally, *P. lobata* had higher relative abundance of Oceanospirillales, harbouring the potential symbiont, *Endozoicomonas* (Bayer *et al.* 2013; Neave *et al.* 2016). *M. foliosa* harboured

Oceanospirillales as well, but at a lower relative abundance, and differed from *P. lobata* in that it contained Oceanospirillaceae instead of Endozoicimonaceae. Oceanospirillaceae has been found to dominate the *Porites* genera (Speck and Donachie 2012), and may be playing a similar symbiotic role as Endozoicimonaceae as they are commonly found in corals. *M. foliosa* in the low disturbance level had more Myxococcales, bacteria suggested to be part of the core biota for *Mussismilia braziliensis* (Garcia *et al.* 2016) and may play a role in corals through sulphate reduction or organic waste decomposition (Lawler *et al.* 2016). Corals in the high disturbance level had higher amounts of Endozoicimonaceae, Rhodobacteraceae, and Vibrionaceae, a consortium of potential symbionts, opportunists, and potential pathogens (Meron *et al.* 2011; Bayer *et al.* 2013; Frydenborg *et al.* 2013), thus potentially playing opposing roles for the coral.

Microbial communities differed significantly between sampling time points (i.e. between the pre-bleaching and bleaching hotspot). Endozoicimonaceae increased to > 90% relative abundance in three coral colonies of *P. lobata* in the low disturbance level. Endozoicimonaceae have been negatively correlated with bleaching pathogens (Pantos *et al.* 2015), thus they may play a protective role for the coral. In the low disturbance level, *M. foliosa* had decreases in the amount of Burkholderiales (13% to 4% relative abundance), Firmicutes (12% to 4% relative abundance), and Rhodobacteraceae (12% to 4% relative abundance). Firmicutes are a broad phylum with hypothesized roles ranging from producing antimicrobial compounds to protect the coral host (Shnit-Orland and Kushmaro 2009) to being found on black band diseased corals (Frias-Lopez *et al.* 2004). As stated previously, Burkholderiales are hypothesized to be nitrogen fixers (Morrow *et*

al. 2012) and Rhodobacterales to be opportunistic taxa (Welsh *et al.* 2015), thus potentially playing conflicting roles in the coral microbiome.

3.4.5 Environmental Differences Between Human Disturbance Levels

The coral microbiome could be influenced by the amount of algae and coral cover at each disturbance level. The low disturbance level had higher coral, higher CCA but lower algal cover. Algae produce different DOM (dissolved organic matter) than corals, which is enriched in DNS (dissolved combined neutral sugars) and selects for less bacterial diversity with increased virulence factors in the water column. On the other hand, corals exude DOM similar to the water column and select for high bacterial diversity dominated by taxa from Alphaproteobacteria and few virulence factors (Nelson *et al.* 2013). Thus, the amount of algae versus coral cover on a reef can greatly influence the assemblage of microbes in the water column, and thus microbes that could potentially invade coral microbiome. In support of this, we found these reef water microbial communities were significantly different between human disturbance levels. Thus, coral cover could be driving the differences we found in coral microbiome diversity, with higher coral cover driving a decrease in microbiome diversity. This is supported by Roder *et al.* (2015) where *Endozoicomonas* dominated the coral microbiome in areas with high coral cover, while overall bacterial diversity was higher in areas with low coral cover. Furthermore, *Pseudoaltermonas* has also been demonstrated to induce coral larval metamorphosis on crustose coralline algae (Negri *et al.* 2001; Tebben *et al.* 2015). The reefs at the low impact sites have higher crustose coralline algae cover, thus potentially increasing the amount of *Pseudoaltermonas* in the coral microbiome at the low disturbance site.

Additionally, differences in nutrients (i.e. nitrate and phosphate) could drive differences between the human disturbance levels. Generally, higher nutrients induces coral stress and changes in microbes (Vega-Thurber *et al.* 2009; Vega Thurber *et al.* 2013). However, high impact sites in our study had lower nutrients than the low disturbance sites. The high coral cover in the low disturbance level could be recycling nutrients in the water column through mechanisms like nitrogen fixation to create a 'biological hotspot', as atolls with higher reef area are characterised by higher chlorophyll-a production and phytoplankton biomass (Gove *et al.* 2016). Additionally, coral mucus shedding provides a mechanism for preventing nutrient loss on coral reefs, as it transfers energy and nutrients from the water column to the reef sediment (Wild *et al.* 2004). The high coral cover at the low disturbance level may maximize mucus shedding and the amount of nutrient retention. In opposite patterns, nitrate plus nitrite and phosphate increased at the low disturbance level, however they decreased at the high disturbance level from the pre-bleaching to bleaching hotspot. This may be due to an increased amount of coral bleaching during the bleaching hotspot, thus expelling cells into the water column and increasing the available nutrients. Since the low disturbance site has higher coral cover, there may be more cells being expelled into the water column.

Fish populations may also impact the coral associated microbial communities. Fish supply nutrients to coral reefs (Allgeier *et al.* 2014; Shantz *et al.* 2015) so differences in fish assemblages at high and low disturbance will affect nutrient levels. Additionally, damselfishes indirectly influence the coral microbiome by cultivating filamentous algae, which can cause increases in coral pathogens and disease (Casey *et al.* 2014). Certain fish communities influence the coral microbiome by slowing coral

disease progression (Cole *et al.* 2009) and functionally diverse communities may decrease coral disease (Raymundo *et al.* 2009). As our human disturbance metric is based on fishing pressure, there likely are differences between the fish assemblages at each disturbance level that could then influence the microbial communities in both positive and negative ways.

Furthermore, in both sampling time points, there was significantly higher microbial abundance in the water column at the high versus the low disturbance level. Microbial abundance in the water column has been shown to increase with fish farm effluent (Garren *et al.* 2009) and thus increase the amount of bacteria interacting with the coral surface. However, corals can shed bacteria from their surface layer (Garren and Azam 2011), thus providing corals in the high disturbance level a way to adapt to higher amounts of bacteria in the water column. Albeit, this mechanism may mean the coral is allocating energy towards mucus shedding rather than other life-history strategies (e.g. reproduction or growth).

3.4.6 Differences in Microbial Community Composition Between Coral Species

Prior to bleaching, each coral species harboured significantly different microbial communities. Vibrionales were consistently in high abundance within *P. lobata.* Vibrionales contain many species associated with disease and bleaching (Bourne *et al.* 2007; Frydenborg *et al.* 2013; Tout *et al.* 2015) but are also found on healthy corals (Lee *et al.* 2012; Rubio-Portillo *et al.* 2014) and contain species that are potential coral symbionts (Shnit-Orland *et al.* 2012). In addition, Endozoicimonaceae, a family with common potential coral symbionts (Bayer *et al.* 2013; Blackall *et al.* 2015; Neave *et al.* 2016) was commonly found within *P. lobata* (~10% relative abundance). In contrast, *M.*

foliosa was composed of Rhodobacterales, potentially opportunistic taxa that have been found on both healthy and disease corals (Meron *et al.* 2011; Sharp *et al.* 2012) and may increase in abundance after coral pathogens open niche space (Welsh *et al.* 2015). *M. foliosa* also contained Burkholderiales, potential nitrogen fixers (Morrow *et al.* 2012) that have shown conflicting associations with coral disease (Cárdenas *et al.* 2012). Therefore, further examination of the relative benefits and stresses caused by Burkholderiales in *M. foliosa* is warranted.

M. foliosa and *P. lobata* differ in their life history strategies and potential resistance capability to stressors, thus possibly influencing their microbiome. *P. lobata* is a "weedy" slow growing coral suggested to be more resistant to stressors, whereas *M. foliosa* has "competitive" life history traits, growing quickly and dominating reefs under ideal conditions (Darling *et al.* 2012). *M. foliosa* are, however, also susceptible to stress events, and may fall in a more "generalist" strategy, dominating in areas with lower competition due to low levels of stress (Darling *et al.* 2012). Thus, as a weedy coral, *P. lobata* may allocate more energy towards regulating its microbial community, versus *M. foliosa* which may allocate more energy towards growth and competition, thus lowering the alpha diversity compared to *M. foliosa* by being less selective. Additionally, *M. foliosa* is a plating coral, the surface area to volume ratio is higher, thus having more area for potential bacteria to invade the coral, and increasing alpha diversity. Future studies could address this question by comparing the alpha diversity across coral life history strategies and morphologies.

3.4.7 Caveats of the Study

Our study would be stronger with more sites within each human disturbance level, thus allowing us to utilize site as a random effect or a step further, use Bayesian hierarchical models (Lunn *et al.* 2000). In lieu of this, we were able to include site as a fixed nested effect, but more sites within each disturbance level would likely strengthen our results. Furthermore, some of our statistical analyses did not allow inclusion of a random effect or even multiple fixed effects. Additionally, any 16S primer is limited, and primers need to be continuously updated and evaluated as discovered microbial diversity increases (Winsley *et al.* 2012). For example, the Earth Microbiome Project recently updated their primers as they discovered their old primers (515F/806R) missed the majority of the most common bacteria in the ocean, SAR11 (Apprill *et al.* 2015). Therefore, there is likely even more bacterial diversity that is missed by primers. Finally, adding a positive control in our PCRs would have given us more insight into the potential contaminant OTUs within our negative controls.

Moving forward, our understanding of coral microbiome responses to stressors would benefit from evaluating microbial functional diversity instead of taxonomic diversity, as done in macro-ecological diversity studies on marine algae and reef fish (Arenas *et al.* 2006; Mouillot *et al.* 2014). As taxonomy or phylogenetic similarity does not imply similar function, functional diversity may provide a more meaningful result in how bacteria play a role in stress resistance. Evaluating functional diversity could be attempted through metagenomics or using PICRUST on 16S sequence data (Langille *et al.* 2013). Additionally, future research should continue this work by evaluating how microbial communities change after a stress event, for example evaluating how microbial communities shift to an alternative state (Ainsworth and Gates 2016) or how corals select

their microbial community to adapt to environmental conditions (Reshef *et al.* 2006). Finally, future research should elucidate if and how (i.e. mechanisms) different microbial communities confer resistance to and recovery from stressors continuously threatening coral reefs.

3.5 Conclusions

Our study demonstrates the combined impact of natural local (i.e. human disturbance) and global stressors (i.e. almost 13 DHWs) on coral microbiome community structure. Overall, human disturbance and a bleaching hotspot caused coral species to become more similar and thermal stress also homogenized corals between human disturbance levels. Both human disturbance and thermal stress appeared to decrease coral regulation of its' microbiome by increasing both alpha and beta diversity. This increase in microbial alpha diversity is contrary to other host microbiomes, where low diversity can imply a stressed microbiome in the human gut (Lozupone *et al.* 2012), potentially due to the coral microbiome being more of an open system. However, increased microbial beta diversity is also found in stressed mammalian hosts (Charlson *et al.* 2010; Moeller *et al.* 2013), thus suggesting a common phenomenon of microbiome destabilization under stress. Our study provides a foundation for understanding the importance of changes in microbial community structure for stressed corals, which is critical for the ongoing catastrophic threats facing coral reefs today (Hughes *et al.* 2003; Pandolfi *et al.* 2003; Hoegh-Guldberg *et al.* 2007; Wiedenmann *et al.* 2013; Ainsworth *et al.* 2016).

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Figures

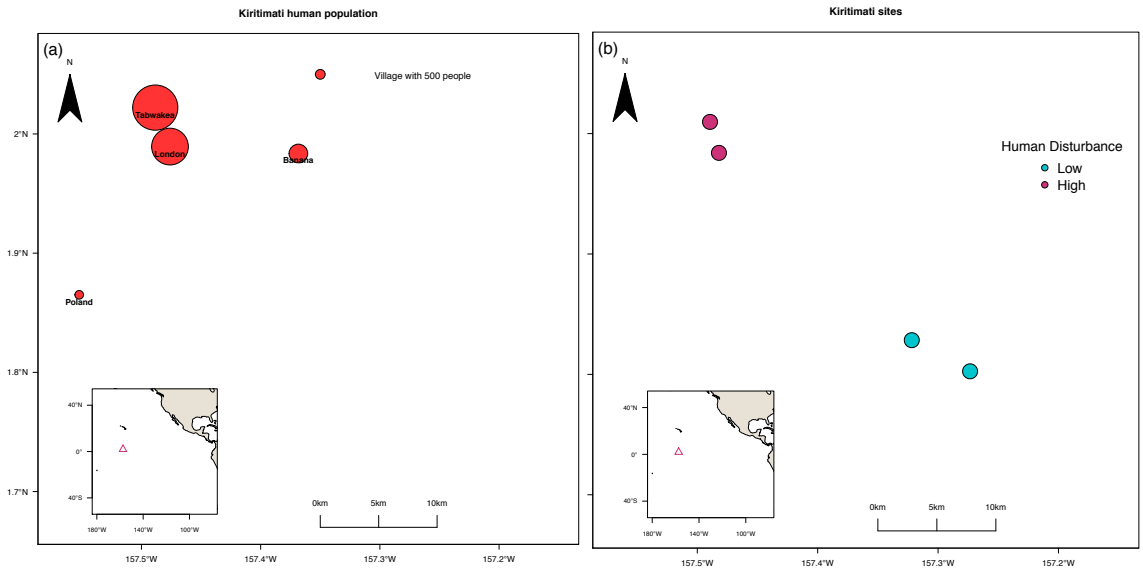

Figure 3.1. Map of Kiritimati (Christmas Island) and (a) villages with bubble size representing number of people and (b) sampling sites in low (turquoise) and high disturbance (pink).

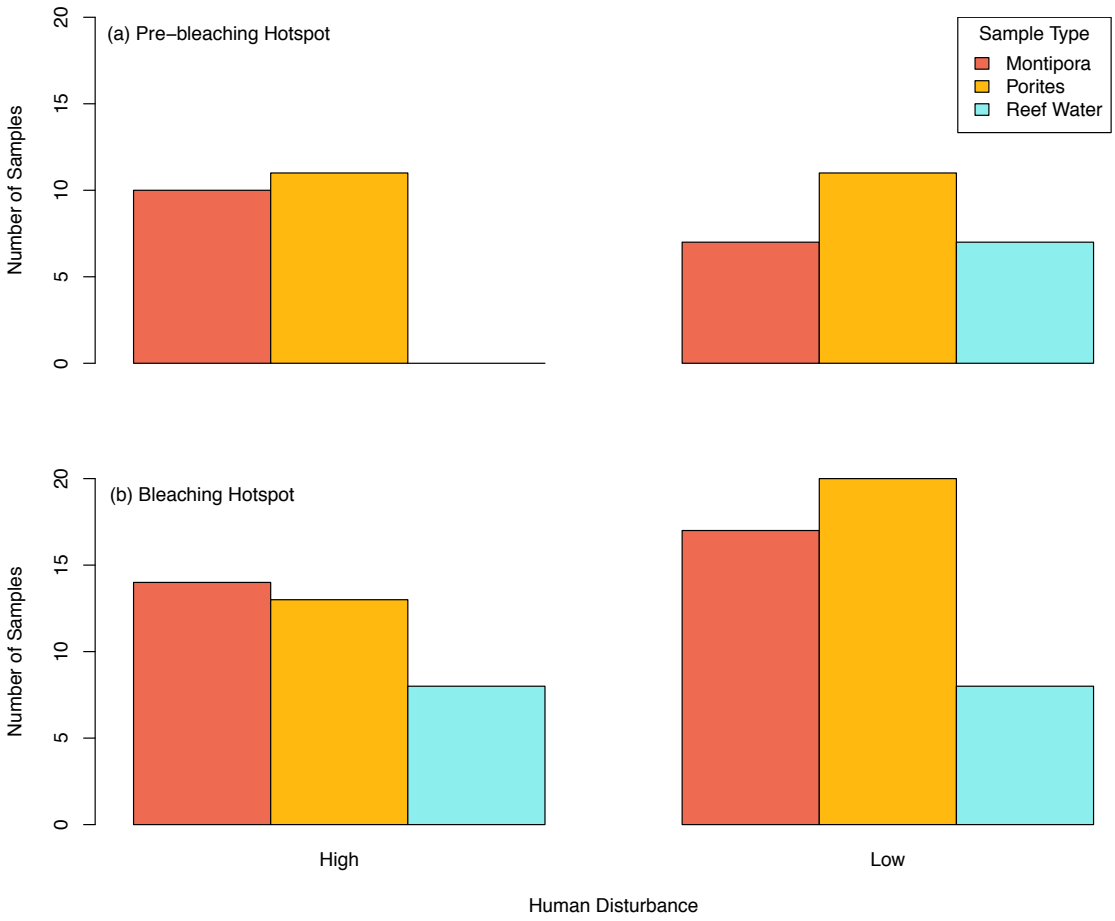

Figure 3.2. Sample size for the (a) pre-bleaching and (b) bleaching hotspot for the sample type (i.e. *Montipora foliosa*, *Porites lobata*, reef water) in each disturbance level.

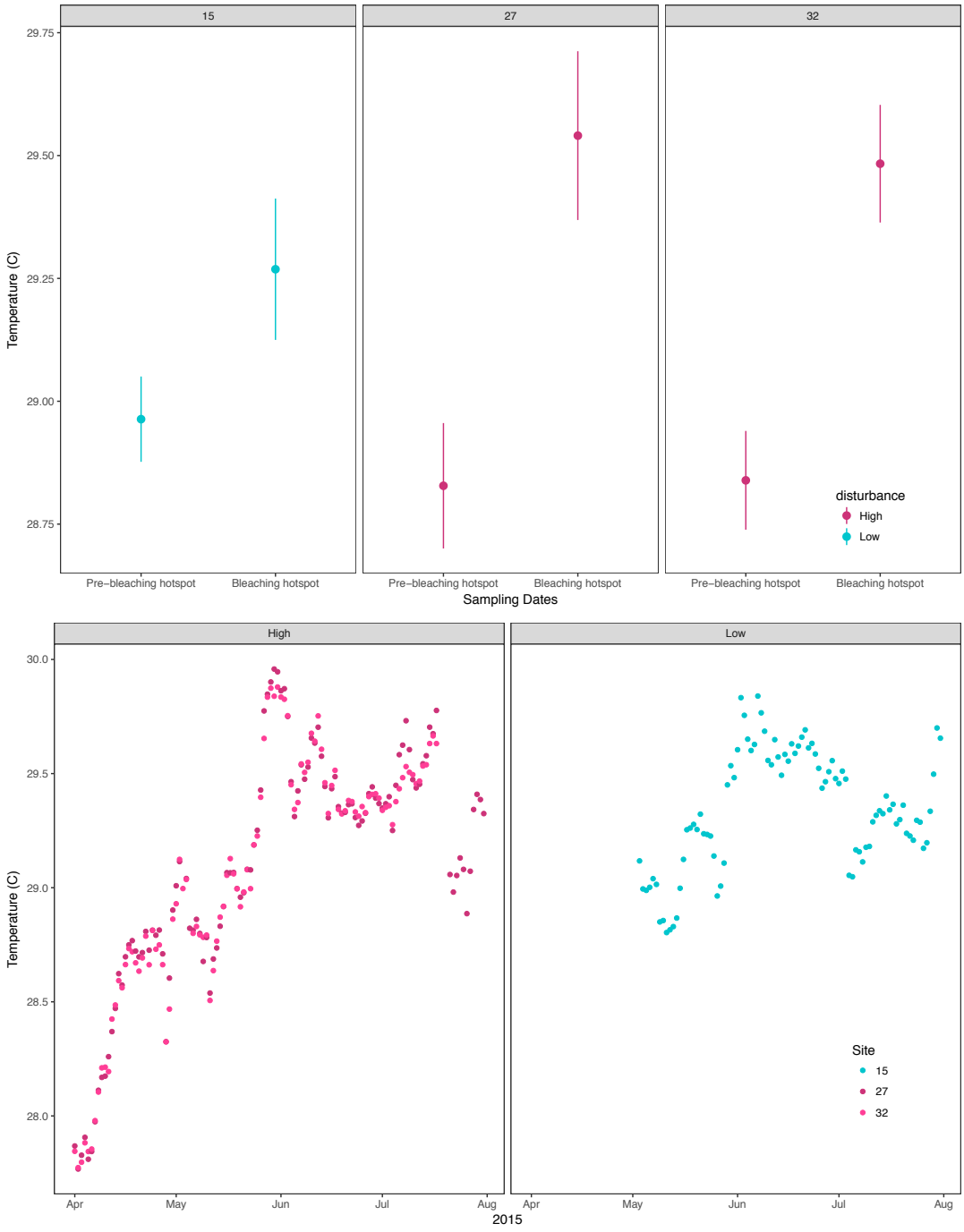

Figure 3.3. Temperature at Kiritimati during the sampling expeditions between sites in each disturbance level (top). Overall temperature for the high (pink) and low (turquoise) disturbance level from April 2015 to August 2015 (bottom). (pre-bleaching hotspot (April 30-May 10) and bleaching hotspot (July 2-19)). Temperature is plotted by site to demonstrate any site variation.

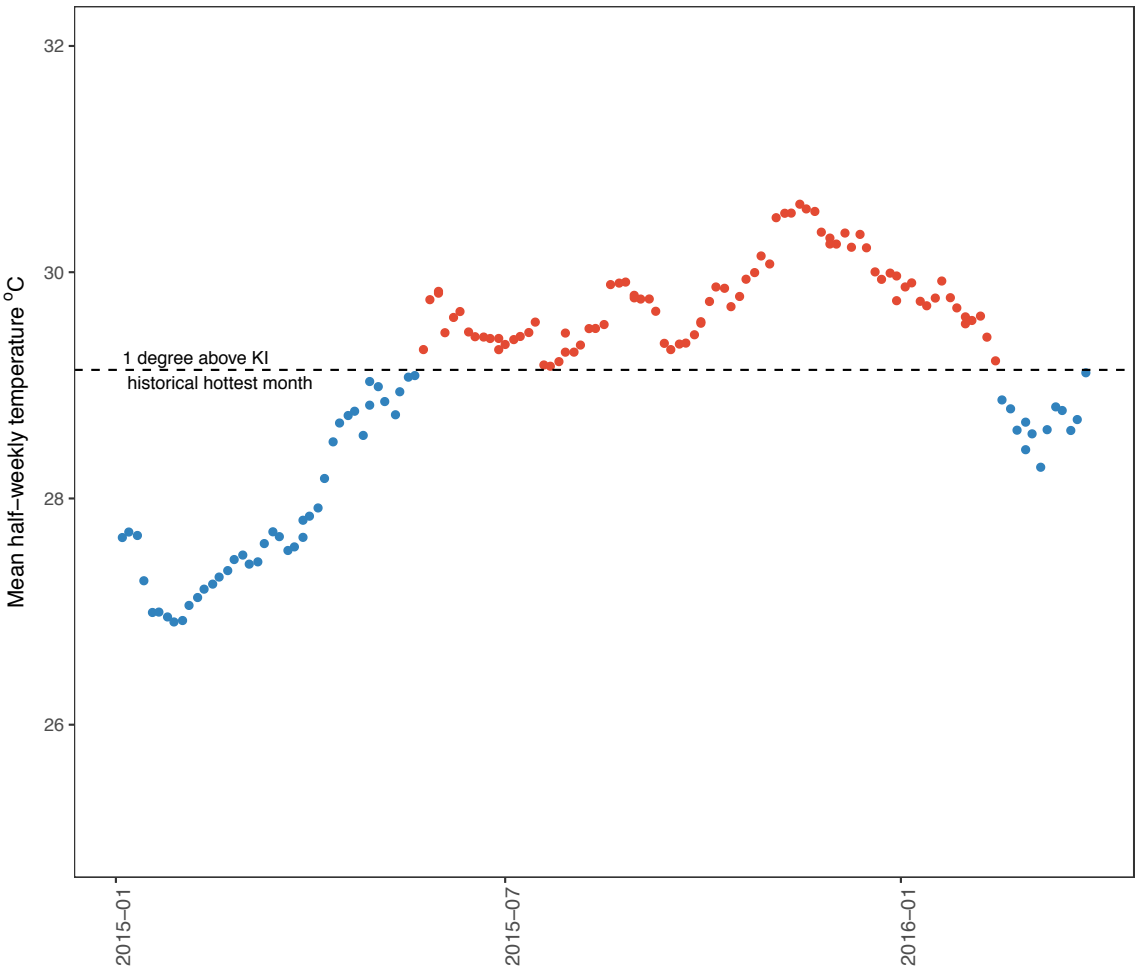

Figure 3.4. Temperature at Kiritimati from January 2015-April 2016. Blue colouring indicates pre-bleaching hotspot conditions where red colouring indicates a bleaching hotspot, where temperatures exceed 1° C over Kiritimati's historical maximum monthly temperature (dashed line) suggesting corals will likely bleach.

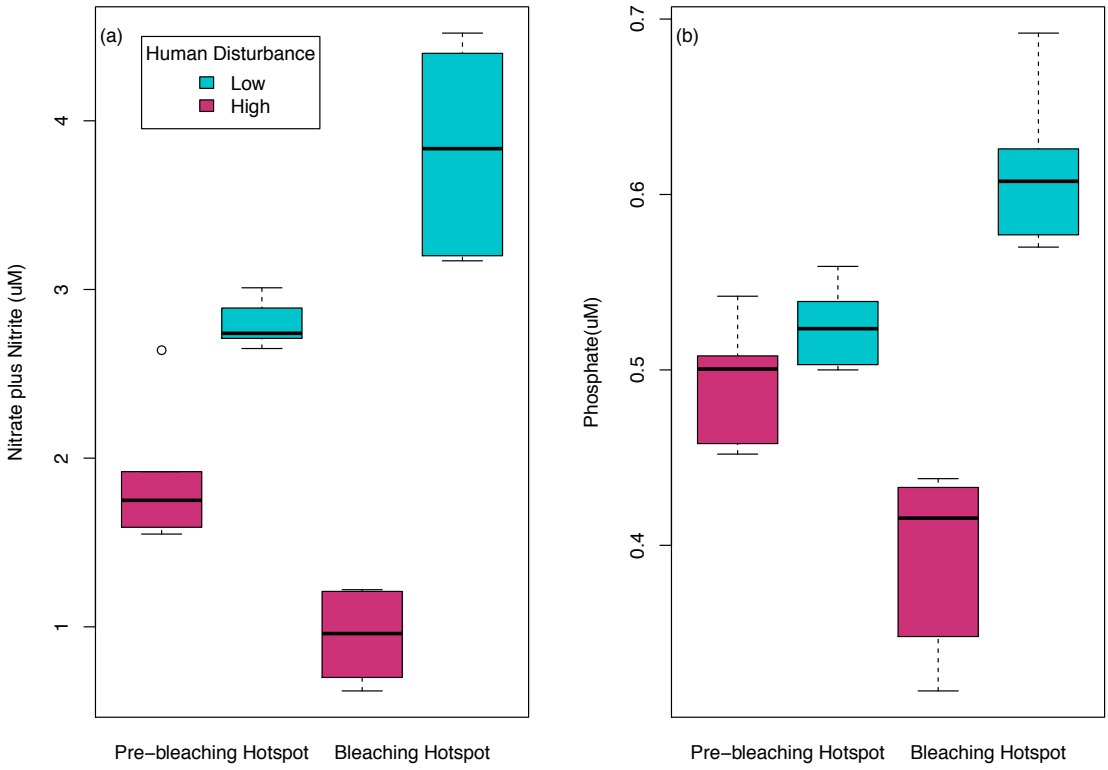

Figure 3.5. Nutrients at Kiritimati for each disturbance level (low=turquoise, high=pink) and sampling expedition (i.e. pre-bleaching hotspot and bleaching hotspot) for (a) nitrate plus nitrite (uM) and (b) phosphate (uM).

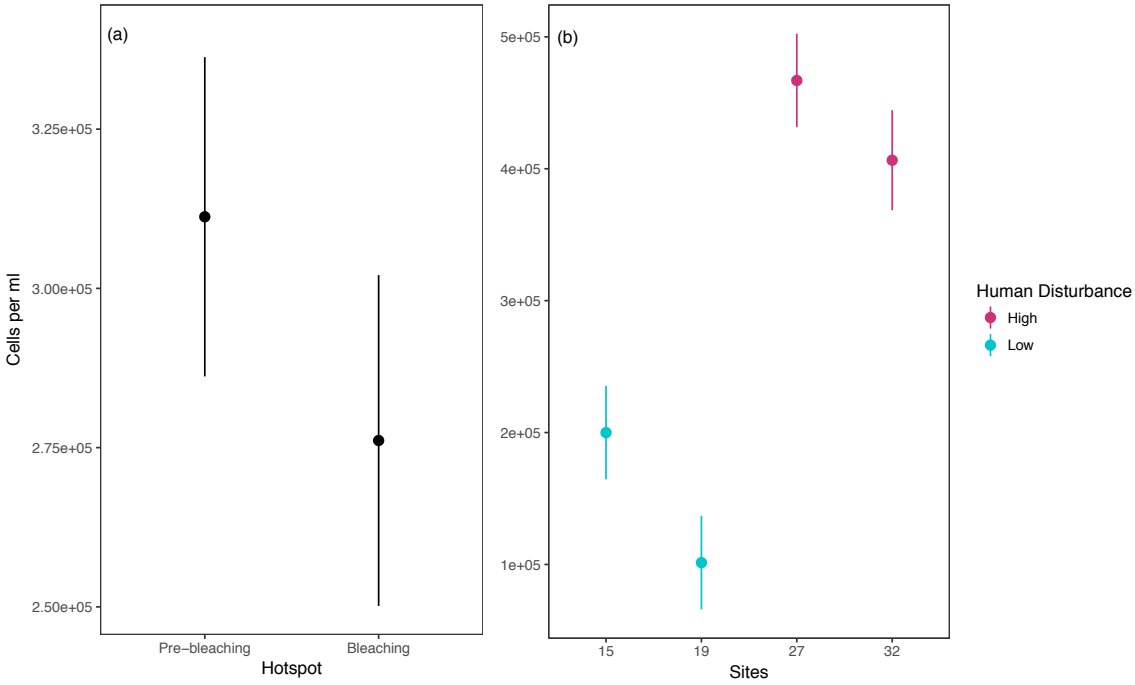

Figure 3.6. Microbial count abundance (cells per ml) estimates from the best model (expedition + human disturbance) during the (a) pre-bleaching hotspot and bleaching hotspot and (b) for each disturbance level (low=turquoise, high=pink). Estimates are plotted by site to demonstrate any site variation.

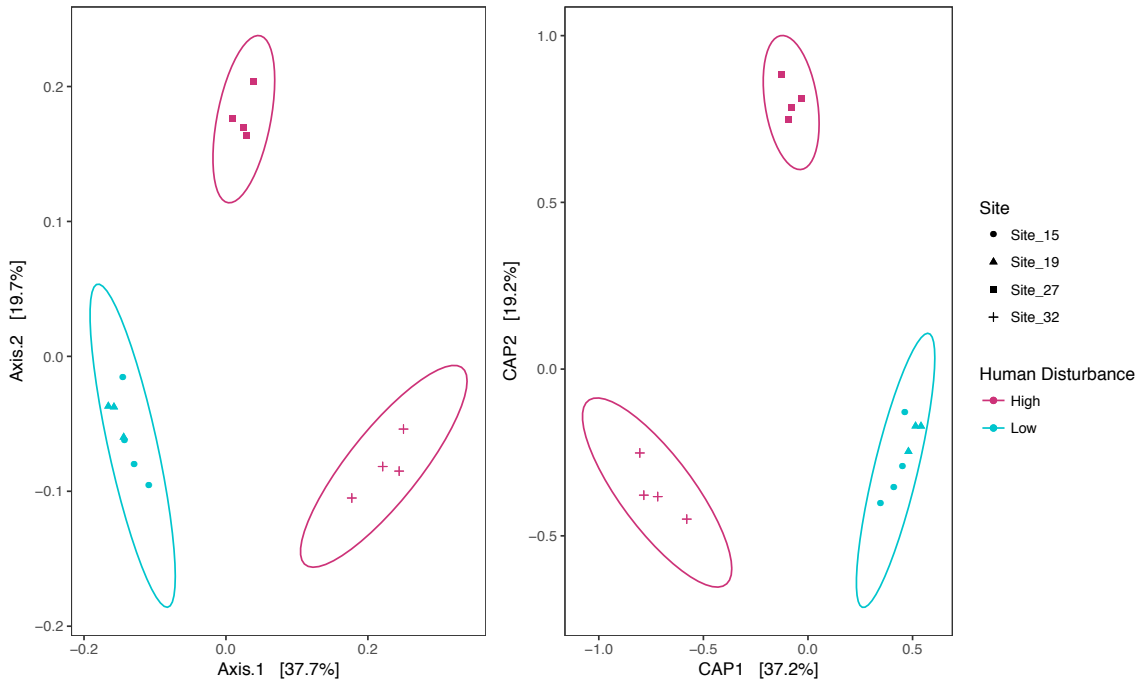

Figure 3.7. (a) Principal coordinates analysis of reef water samples at the high (pink) and low (turquoise) disturbance levels for the bleaching hotspot using Bray-Curtis distance. (b) Canonical analysis of principal coordinates of water samples at the high and low disturbance level, for the bleaching hotspot using Bray-Curtis distance. The ellipses are 95% confidence groupings.

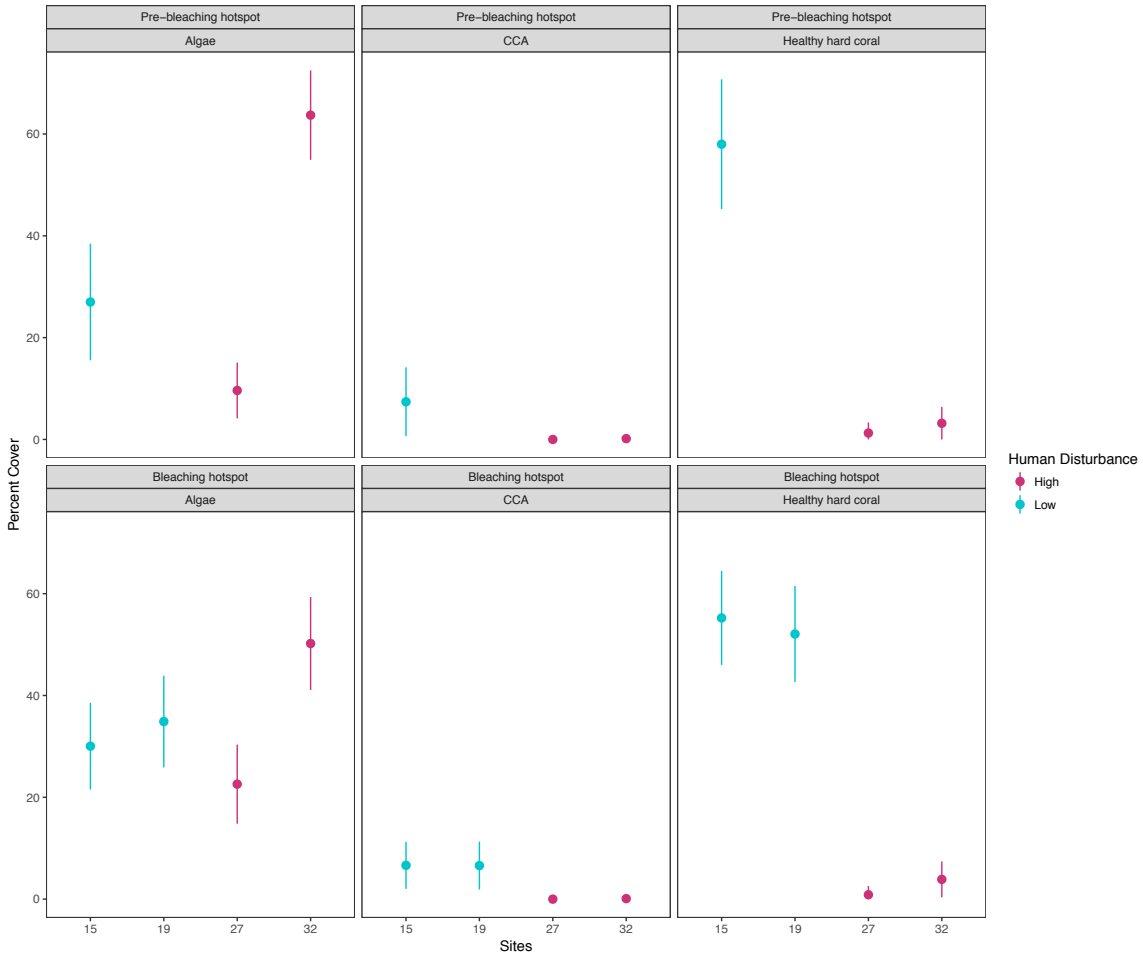

Figure 3.8. Benthic percent cover for the pre-bleaching and bleaching hotspot for algae, crustose coralline algae, and healthy hard coral in the low (turquoise) and high (pink) disturbance level. Error bars represent standard error. Percent cover is plotted by site to demonstrate any site variation.

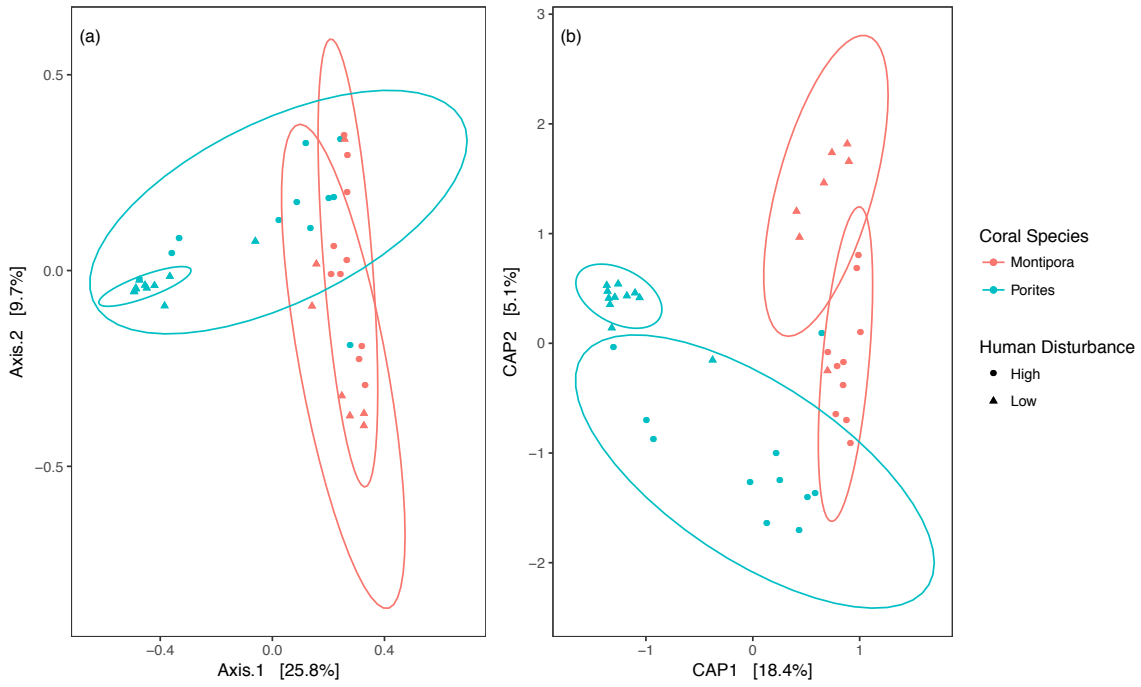

Figure 3.9. (a) Principal coordinates analysis of *Montipora foliosa* (pink) and *Porites lobata* (blue) samples during the pre-bleaching hotspot in both low (triangle) and high (circle) human disturbance using Bray-Curtis distance. (b) Canonical analysis of principal coordinates of *M. foliosa* (pink) and *P. lobata* (blue) samples during the pre-bleaching hotspot in both low (triangle) and high (circle) human disturbance using Bray-Curtis distance (best model after backwards stepwise ANOVA: coral species + human disturbance/site). The ellipses are 95% confidence groupings.

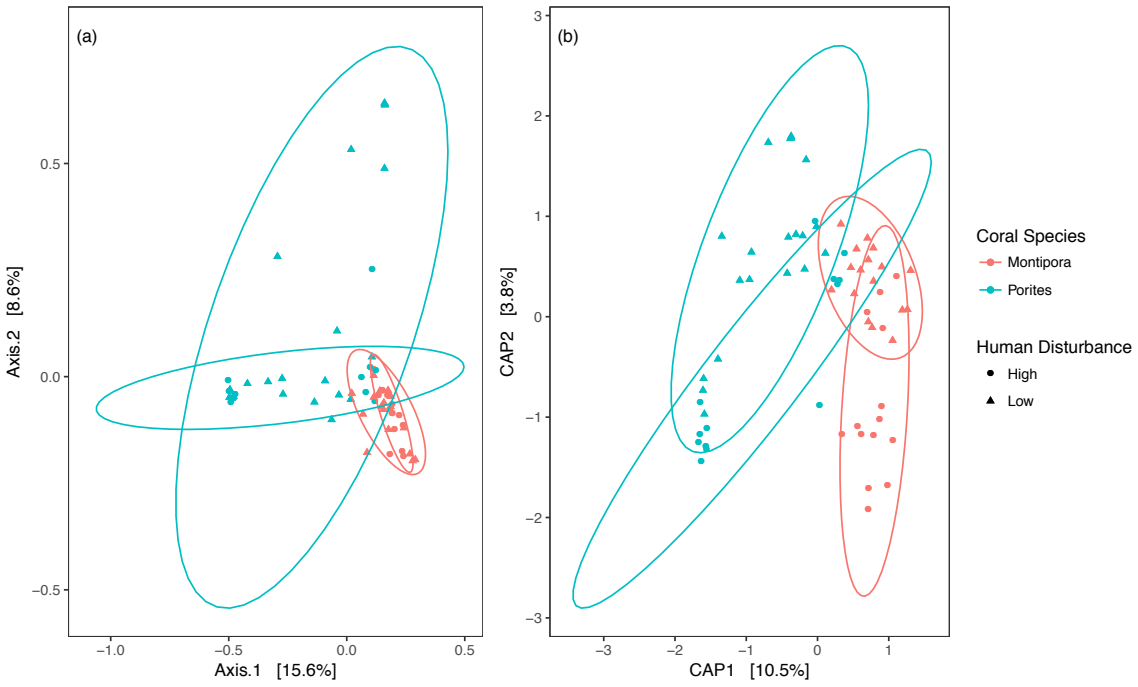

Figure 3.10. (a) Principal coordinates analysis of *Montipora foliosa* (pink) and *Porites lobata* (blue) at the high (circle) and low (triangle) disturbance level, for the bleaching hotspot using Bray-Curtis distance. The ellipses are 95% confidence groupings. (b) Canonical analysis of principal coordinates of *M. foliosa* and *P. lobata* at the high and low disturbance level, for the bleaching hotspot using Bray-Curtis distance (best model after backwards stepwise ANOVA: coral species + human disturbance/site). The ellipses are 95% confidence groupings.

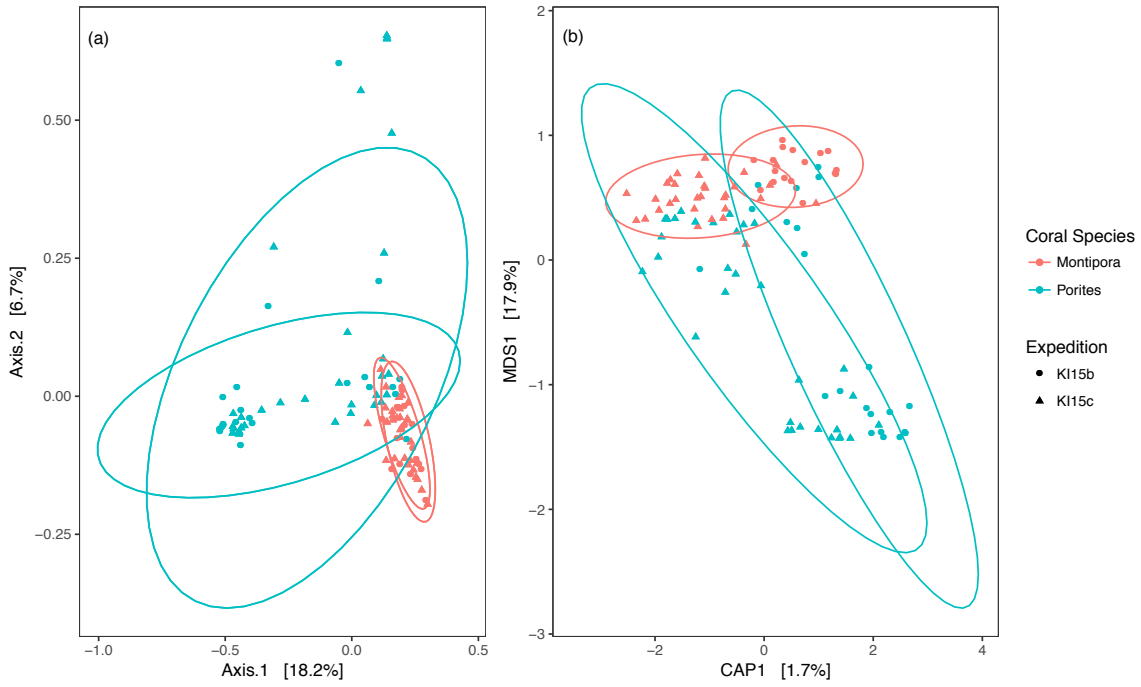

Figure 3.11. (a) Principal coordinates analysis of *Montipora foliosa* (pink) and *Porites lobata* (blue) samples during the pre-bleaching hotspot (circle, KI15b) and bleaching hotspot (triangle, KI15c) using Bray-Curtis distance. The ellipses are 95% confidence groupings. (b) Canonical analysis of principal coordinates of *Montipora foliosa* (pink) and *Porites lobata* (blue) samples during the pre-bleaching (circle, KI15b) and bleaching hotspot (triangle, KI15c) using Bray-Curtis distance (Best model after backwards stepwise ANOVA: expedition while controlling for local colony). The ellipses are 95% confidence groupings.

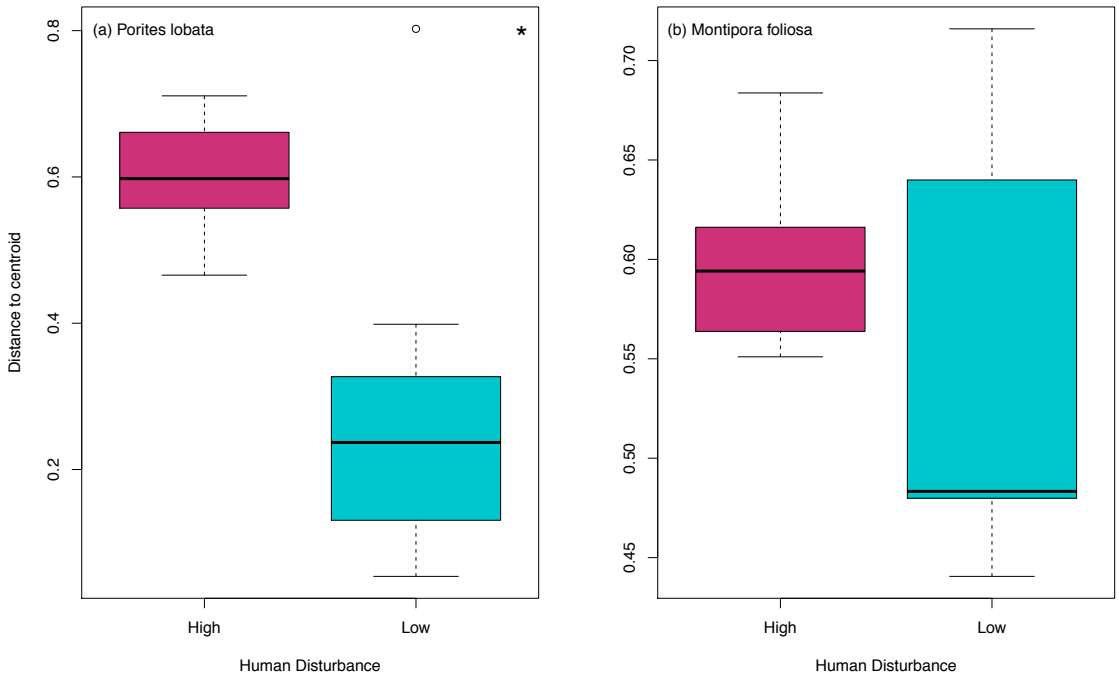

Figure 3.12. PERMADISP (i.e. beta diversity) results for (a) *Porites lobata* and (b) *Montipora foliosa's* microbial communities during the pre-bleaching hotspot between the low (turquoise) and high (pink) disturbance levels.

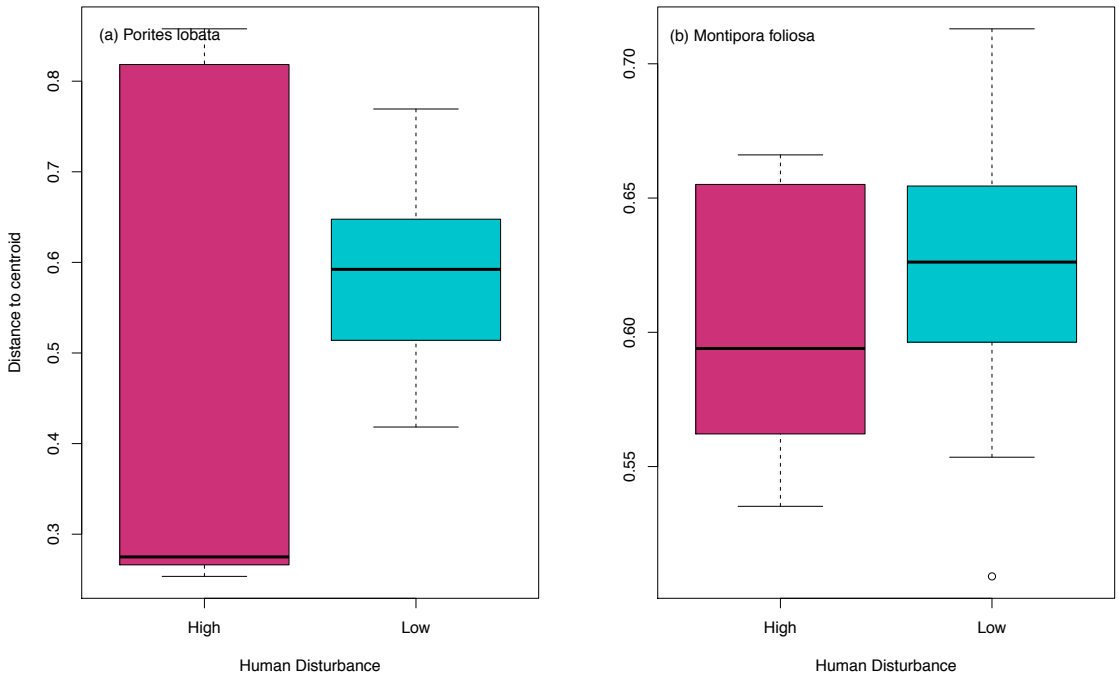

Figure 3.13. PERMADISP (i.e. beta diversity) results for (a) *Porites lobata* and (b) *Montipora foliosa's* microbial communities during the bleaching hotspot for the low (turquoise) and high (pink) disturbance levels.

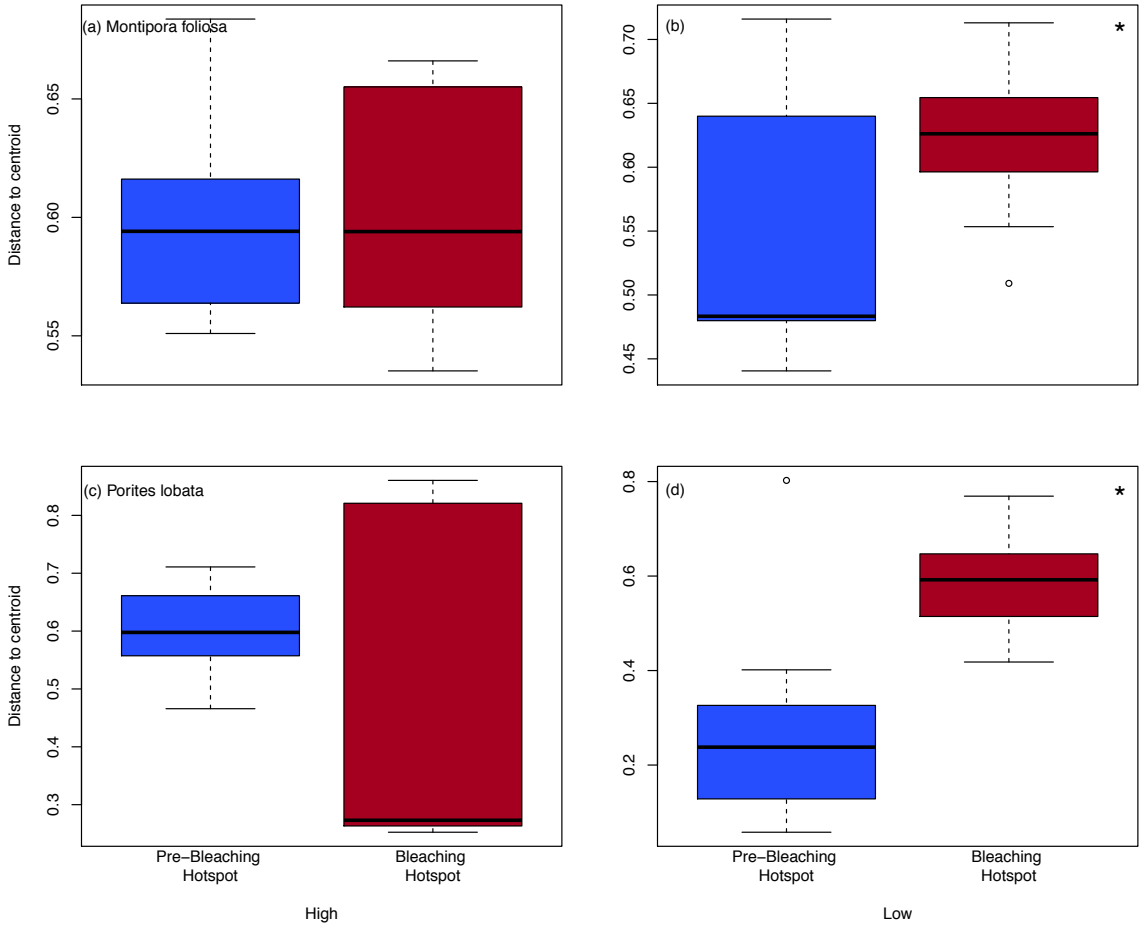

Figure 3.14. PERMADISP (i.e. beta diversity) results for the microbial communities of *Montipora foliosa* in the (a) high and (b) low disturbance and *Porites lobata* in the (c) high and (d) low disturbance level from the pre-bleaching (blue) to bleaching hotspot (red).

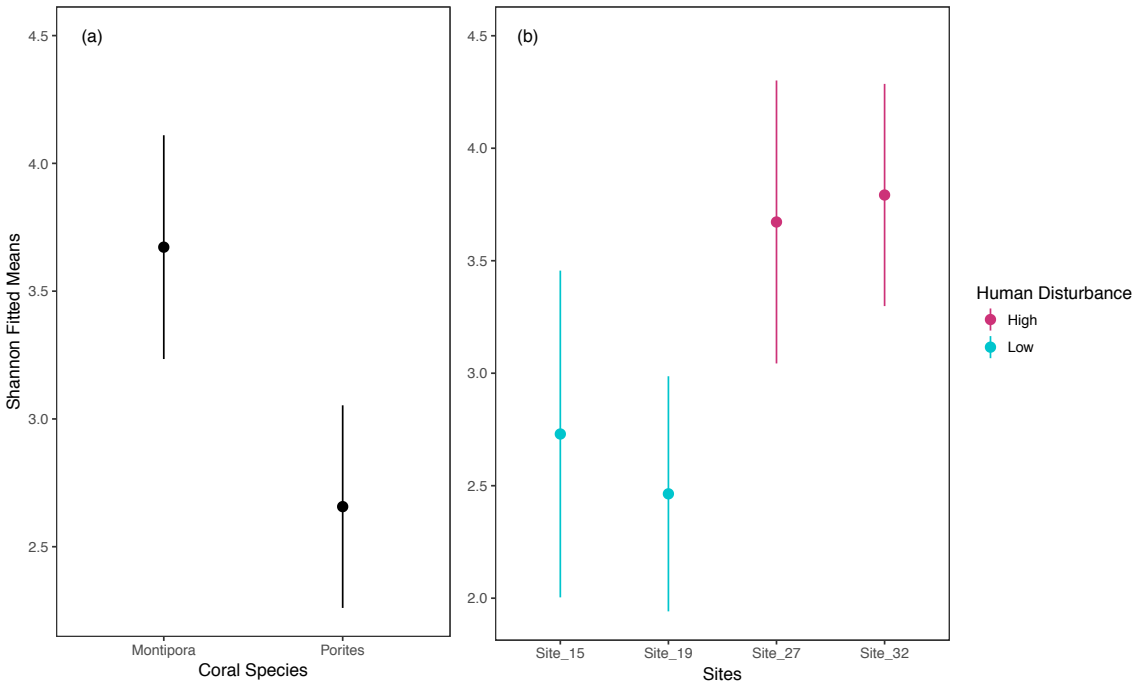

Figure 3.15. Estimated Shannon index means from the best model (coral species + human disturbance) for (a) coral species and (b) human disturbance (low=turquoise, high=pink) in the pre-bleaching hotspot. Estimates are plotted by site to demonstrate any site variation.

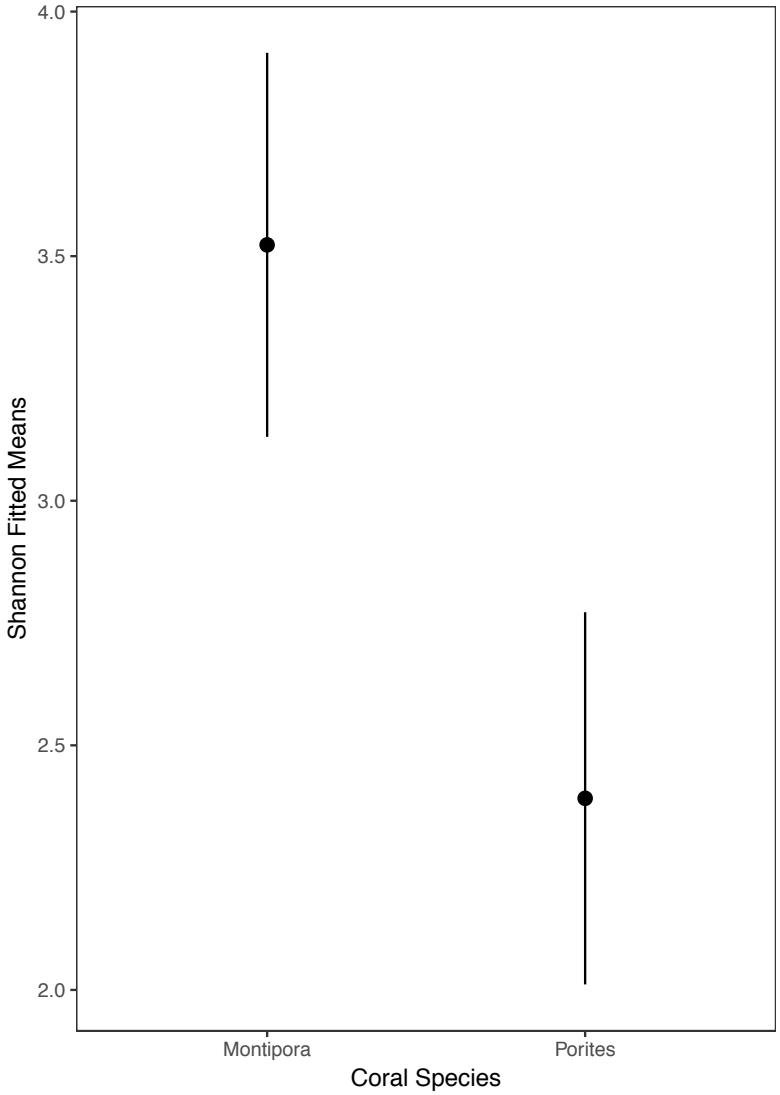

Figure 3.16. Estimated Shannon index means from the best model (coral species) for *Montipora foliosa* and *Porites lobata* in the bleaching hotspot.

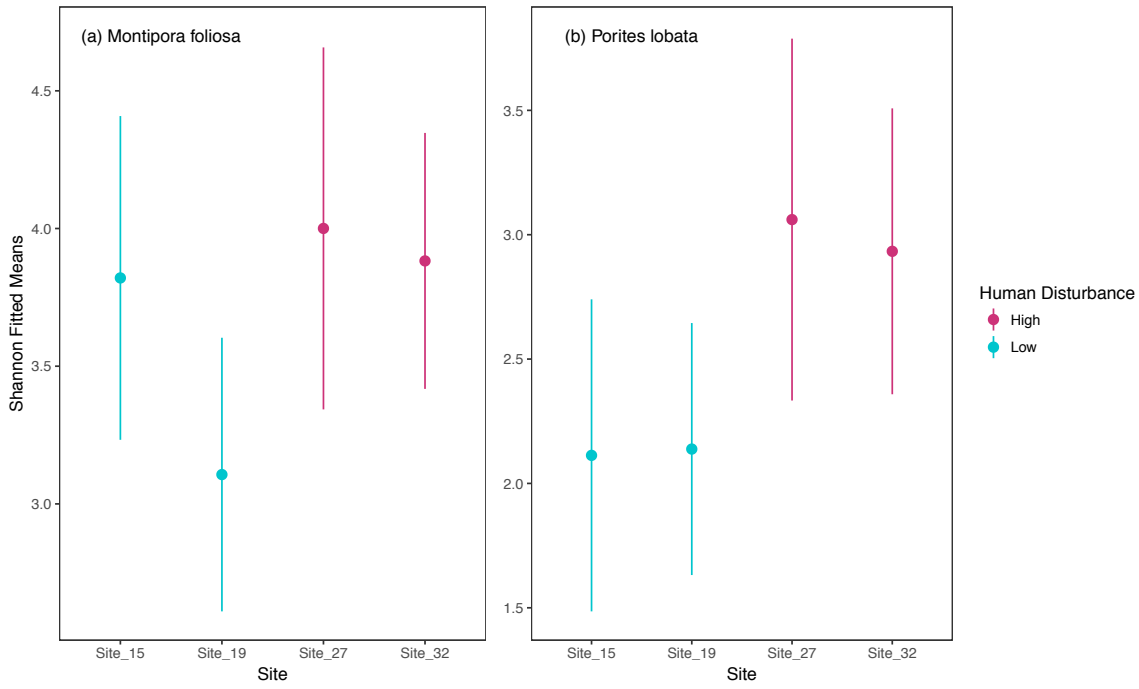

Figure 3.17. Estimated Shannon index means from the best model (human disturbance) for (a) *Montipora foliosa* and (b) *Porites lobata* for all corals combined in the pre-bleaching and bleaching hotspot (high disturbance=pink, low disturbance=turquoise). Estimates are plotted by site to demonstrate any site variation.

Appendix A

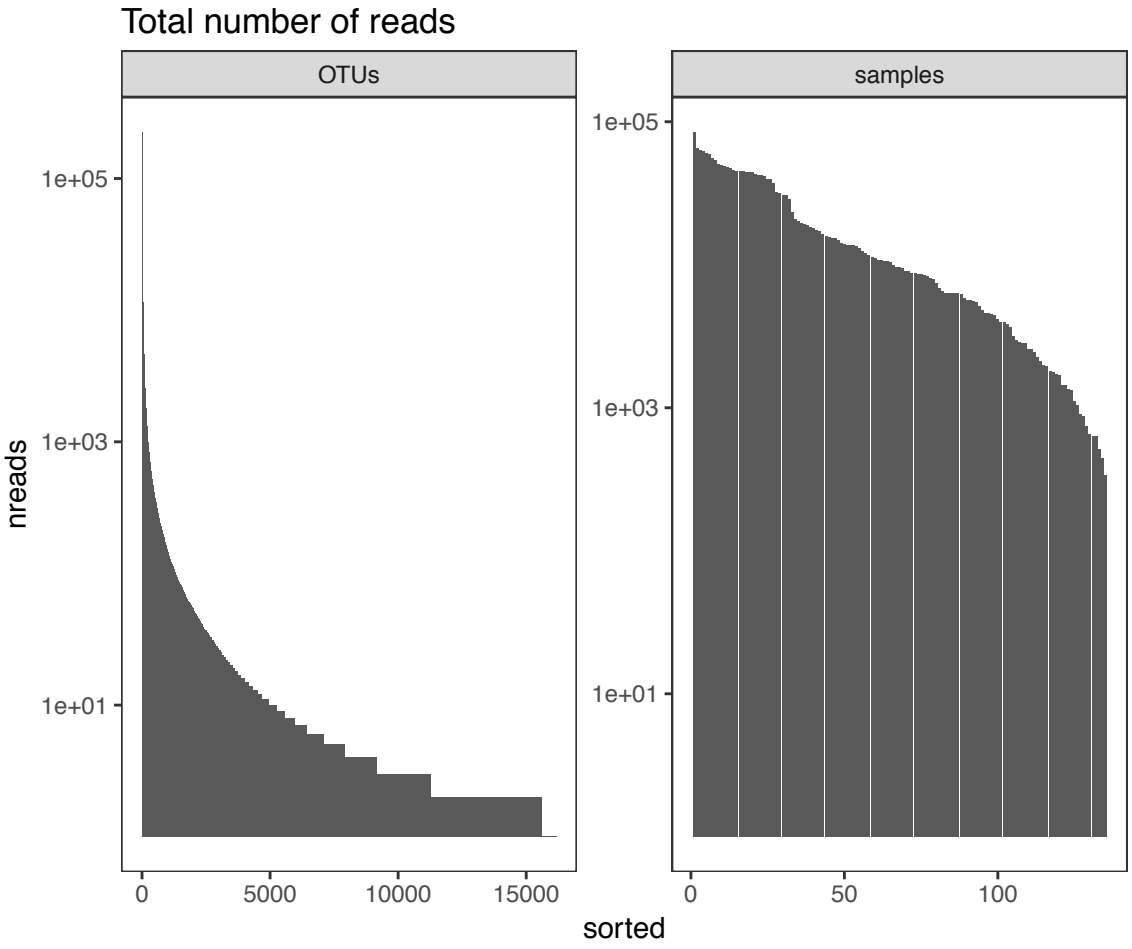

Figure A.1. The total number of reads for each OTU and for each sample (water and coral) prior to contamination filtering but after pre-processing (i.e. filtering out mitochondria and chloroplasts).

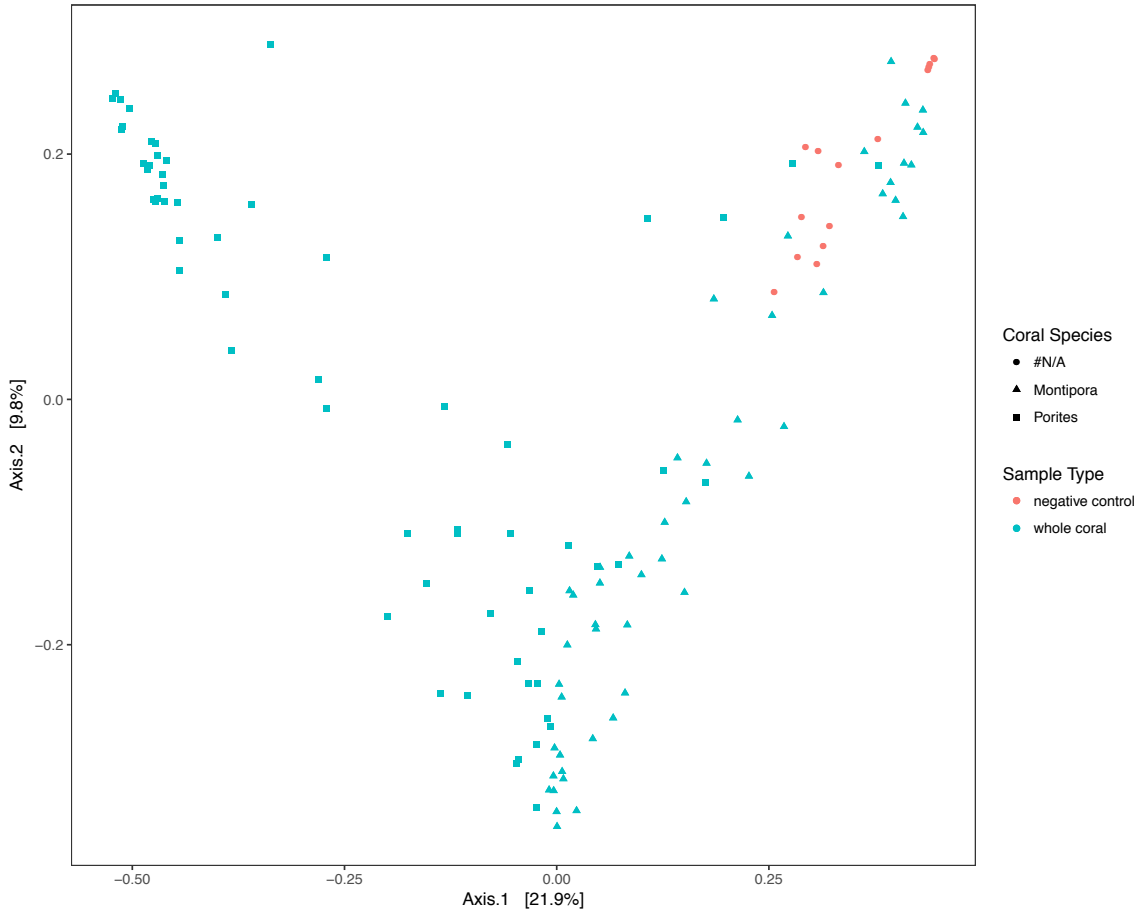

Figure A.2. Principal coordinate analysis demonstrating the similarity of the negative controls (pink) with coral samples (blue) especially *Montipora foliosa* (triangle shape) using the Bray-Curtis dissimilarity index.

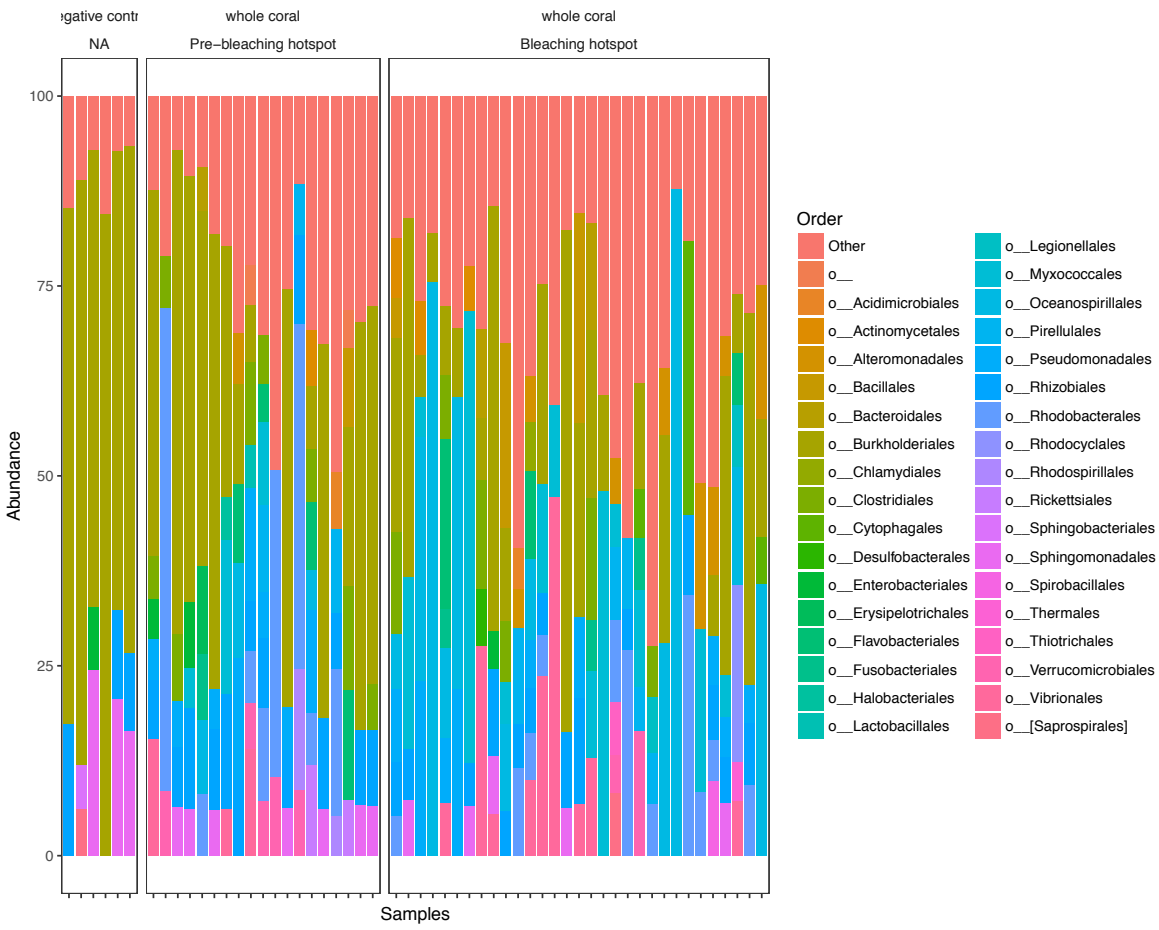

Figure A.3. Bar plot of bacterial relative abundance for the six negative controls and *Montipora foliosa* (i.e. the coral with the highest amount of OTUs similar to contamination). Each bar is a sample. Any orders with less than 5% relative abundance are placed into the "Other" category.

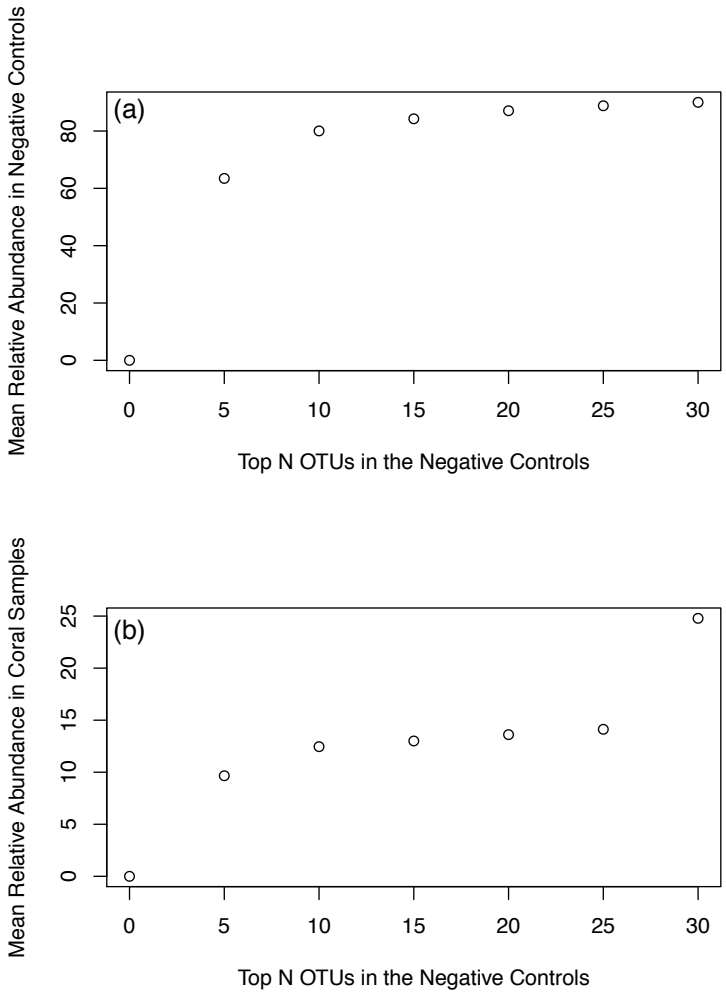

Figure A.4. (a) The mean relative abundance in negative controls of the top 0-30 OTU's found in the negative controls. Note: there were 15 negative controls as there were technical replicates of the negative controls that were sequenced. To determine the average top N OTUs within the negative controls, replicates of the six negative controls were merged. Therefore, there are six unique negative controls. (b) The mean relative abundance in all coral samples of the top 0-30 OTUs found in the negative control samples. Note the plateau starting at N=10 but with a sudden increase at N=30. This sudden increase is due to a single OTU that is highly abundant within coral samples, suggesting it is an important member of the coral holobiont (i.e. OTU 4393354 "k__Bacteria" "p__Proteobacteria" "c__Gammaproteobacteria" "o__Vibrionales" "f__Pseudoalteromonadaceae" "g" "s").

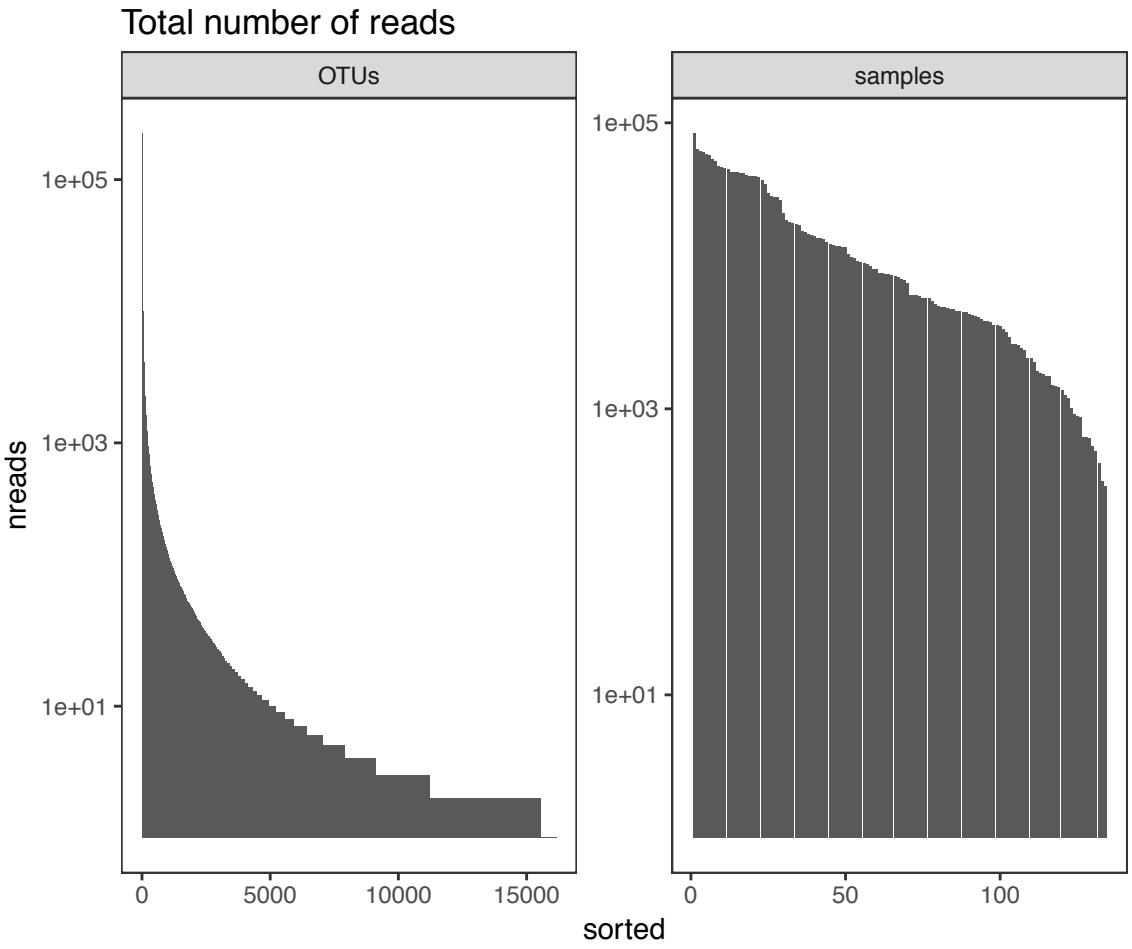

Figure A.5. The total number of reads for each OTU and for each sample after filtering out the top 10 OTUs in the negative controls.

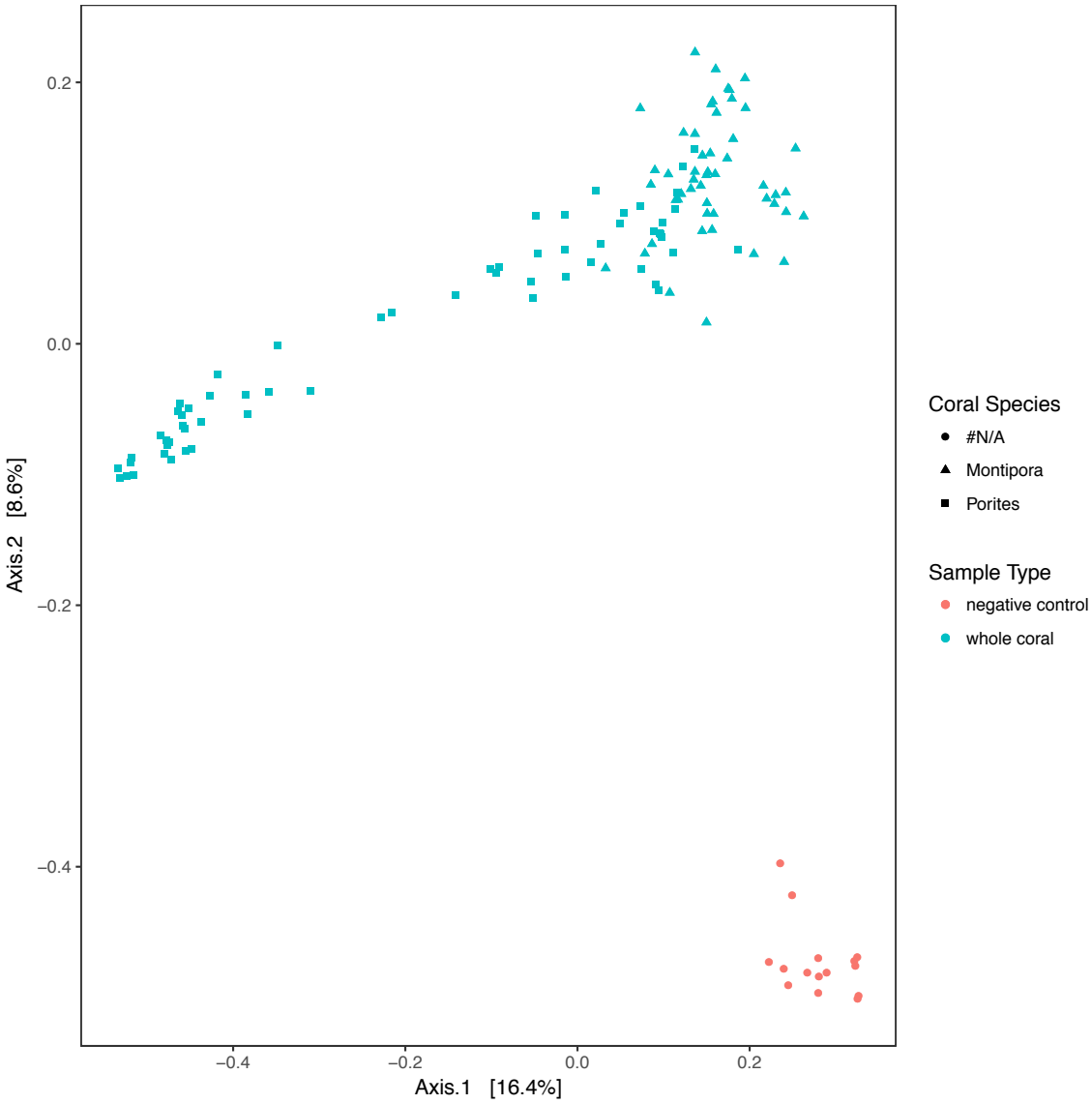

Figure A.6. Principal coordinates analysis demonstrating how filtering the top 10 OTUs found within the negative controls separates the negative controls (pink) from the coral samples (blue) using the Bray-Curtis dissimilarity.

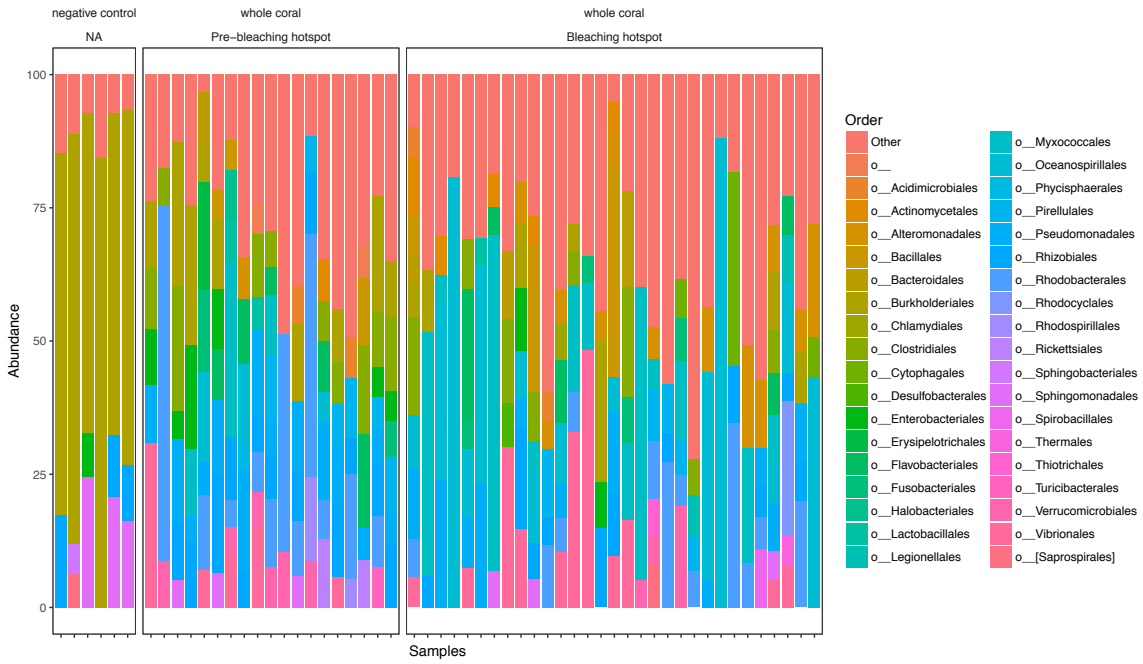

Figure A.7. Bar plot of bacterial relative abundance for the old negative controls and the new *Montipora foliosa* (i.e. with the top 10 OTUs filtered). Each bar is a sample. Any orders with less than 5% relative abundance are placed into the "Other" category.

Table A.1. The top 10 OTUs within negative controls that were filtered out, in order from most abundant to least abundant.

	Kingdom	Phylum	Class	Order	Family	Genus	Species
788519	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	#N/A	#N/A
132704	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	s__
287547	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia	s__
29704	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	g__	s__
1108062	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	s__
147025	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacterium	s__
4336568	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	s__
2279387	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	lividum
254938	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	#N/A	#N/A
3799784	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	g__	s__

Appendix B

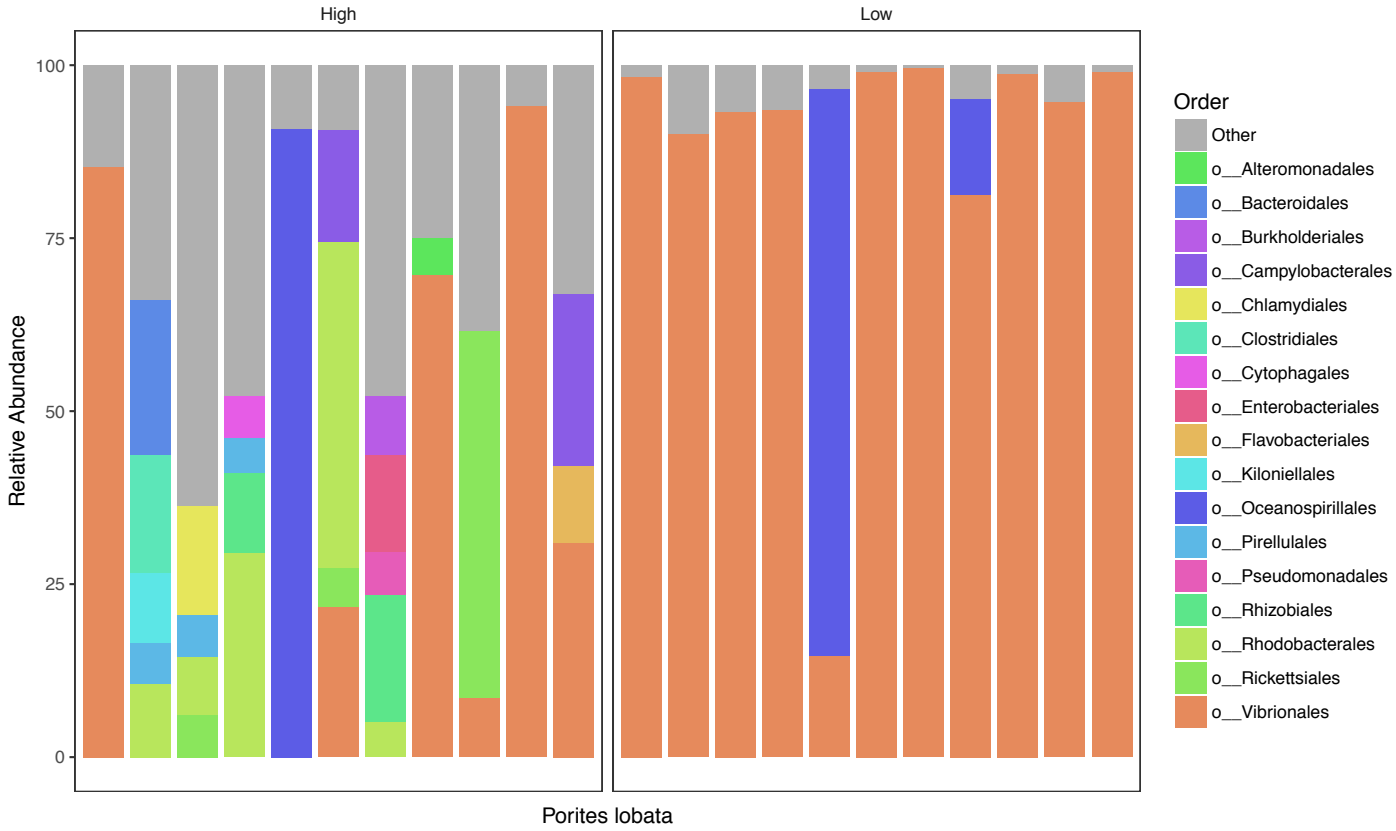

Figure B.1. Relative abundance of microbial orders for each *Porites lobata* sample during the pre-bleaching hotspot in the high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any orders in less than 5% relative abundance.

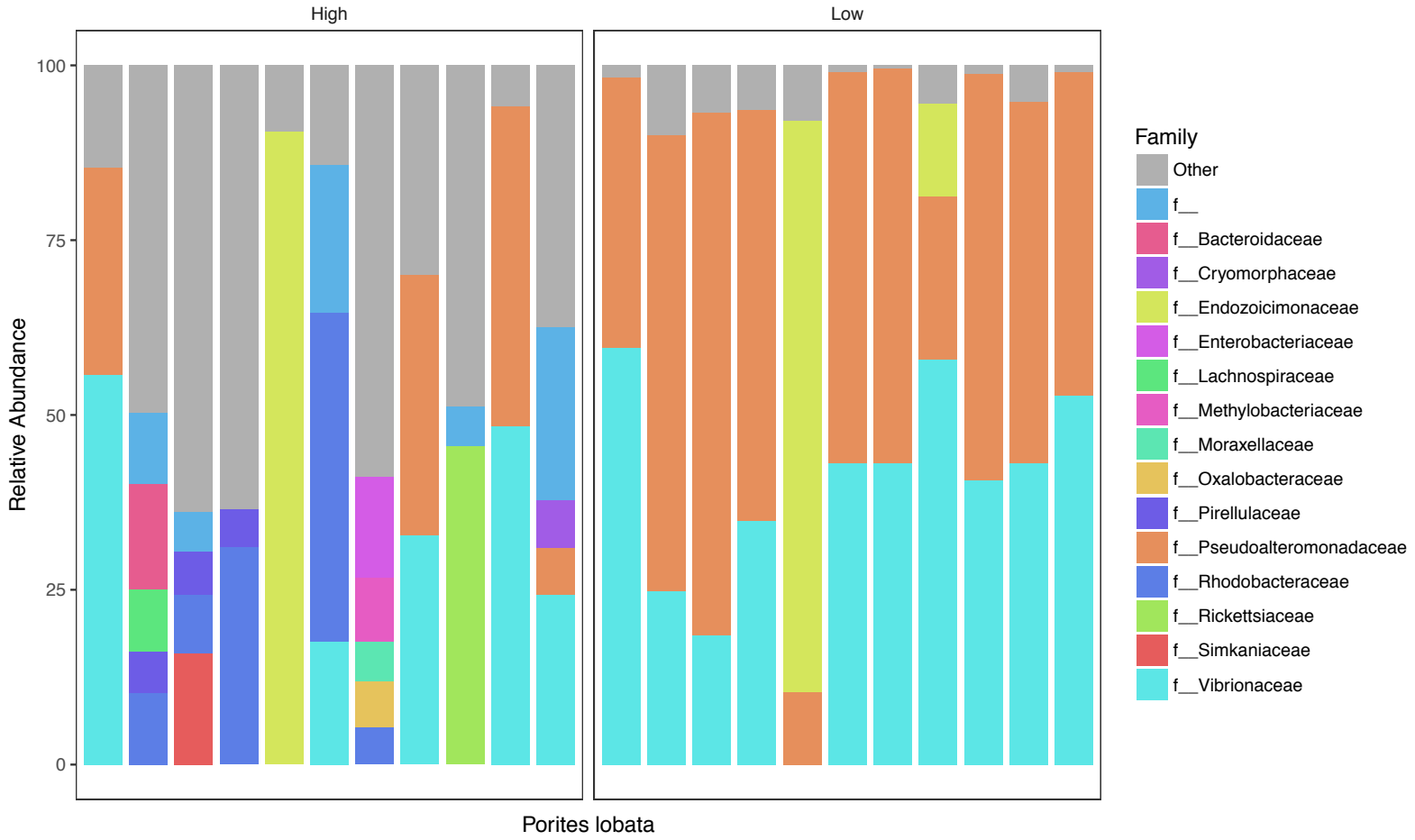

Figure B.2. Relative abundance of microbial families for each *Porites lobata* sample during the pre-bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

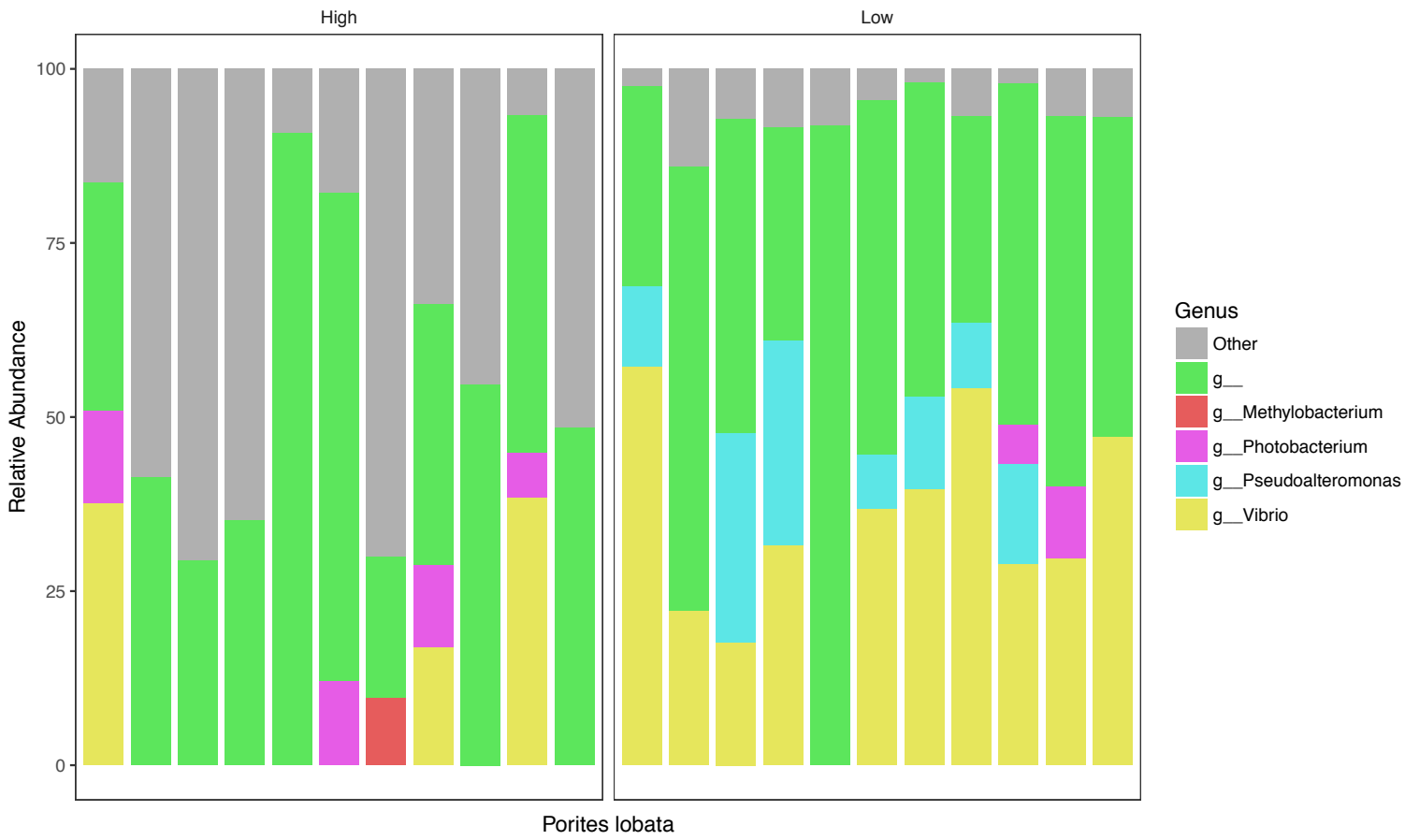

Figure B.3. Relative abundance of microbial genera for each *Porites lobata* sample during the pre-bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

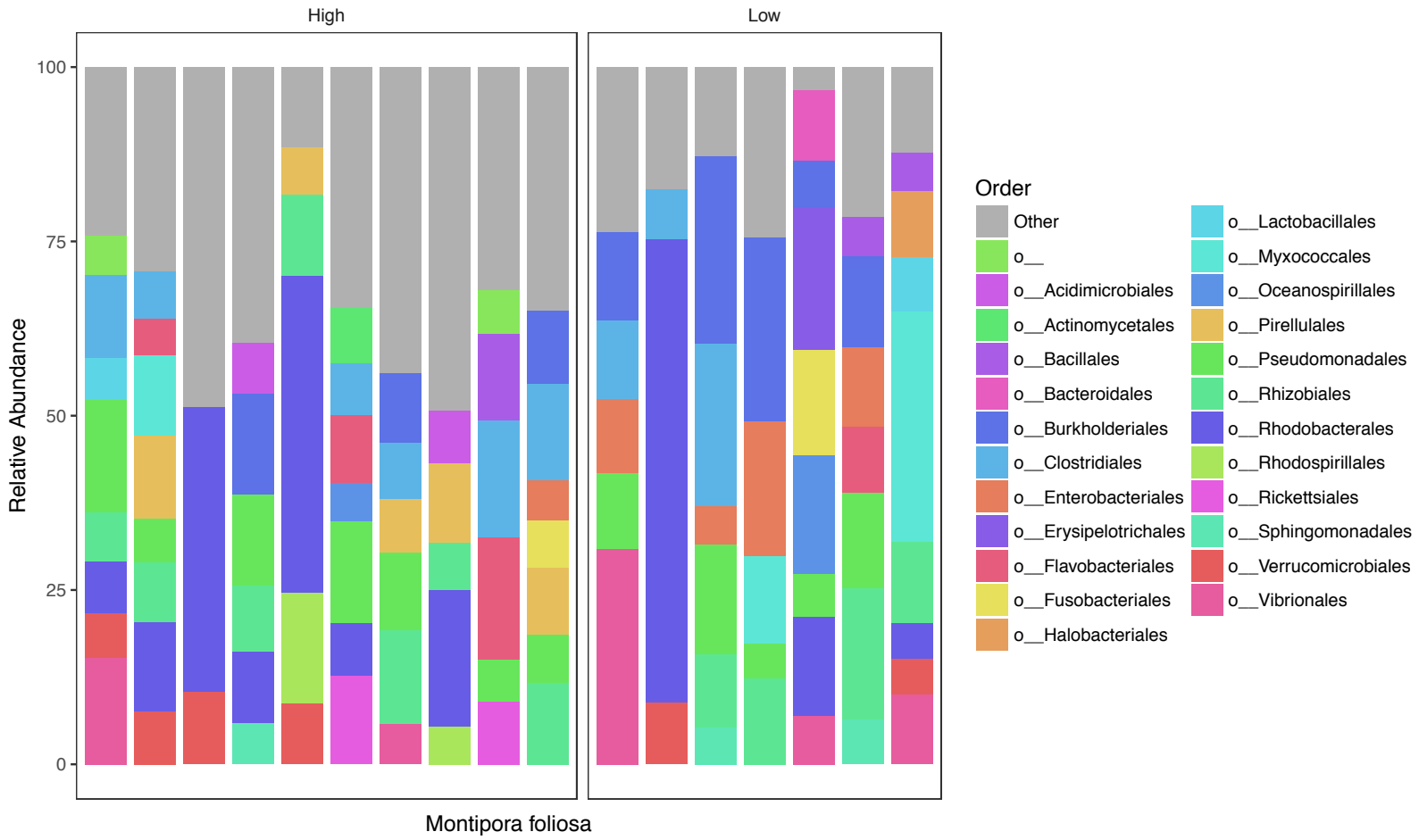

Figure B.4. Relative abundance of microbial orders for each *Montipora foliosa* sample during the pre-bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any orders in less than 5% relative abundance.

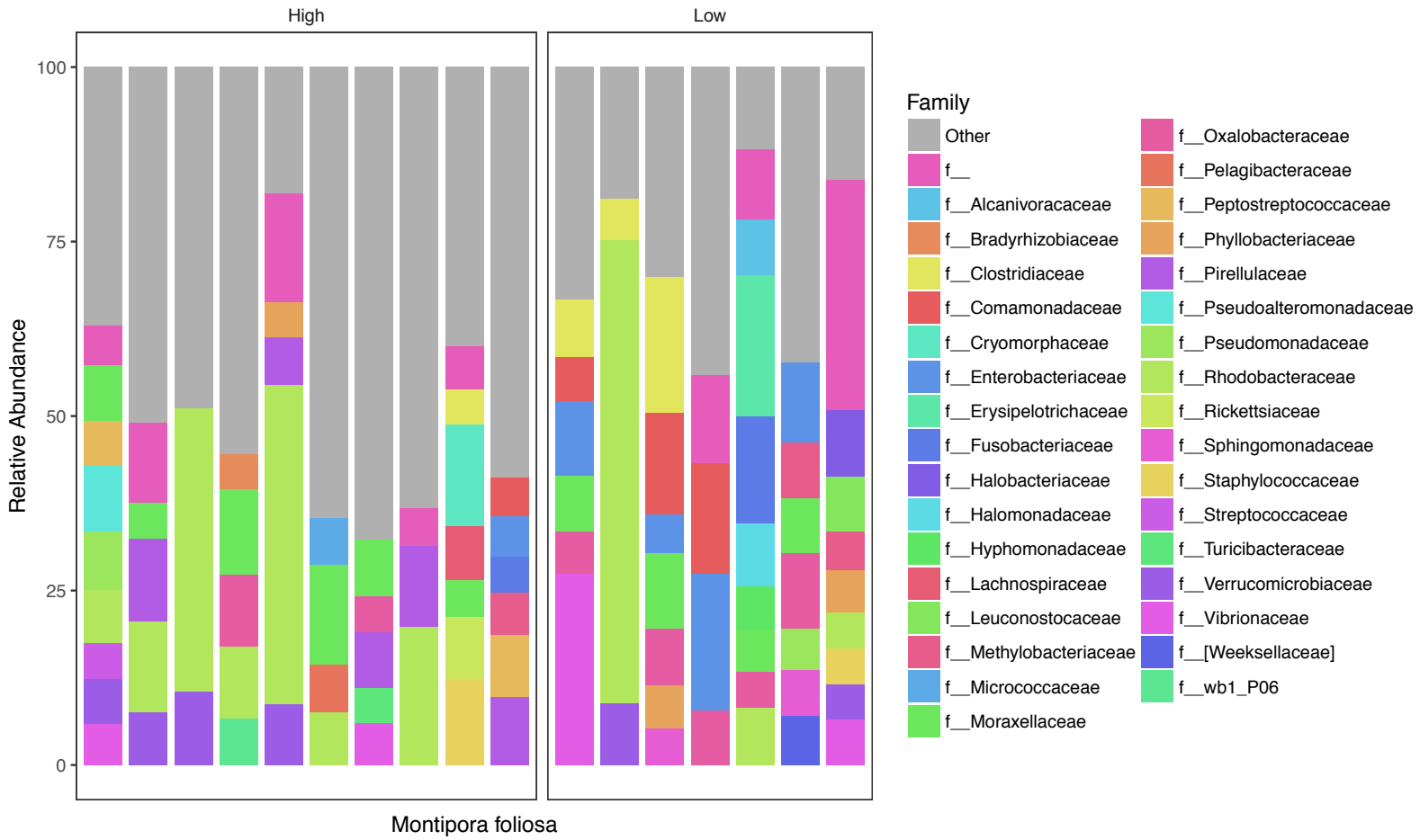

Figure B.5. Relative abundance of microbial families for each *Montipora foliosa* sample during the pre-bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

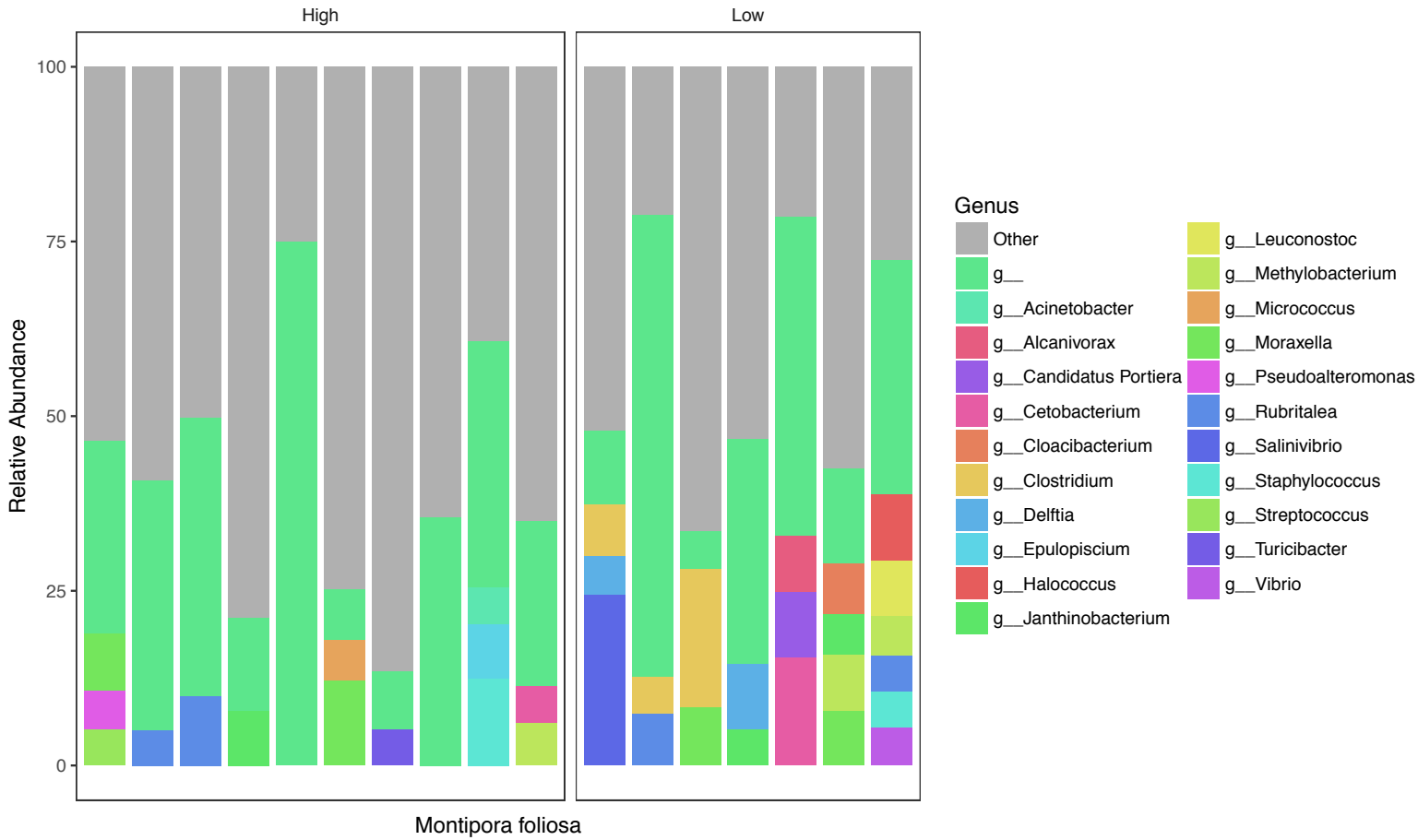

Figure B.6. Relative abundance of microbial genera for each *Montipora foliosa* sample during the pre-bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

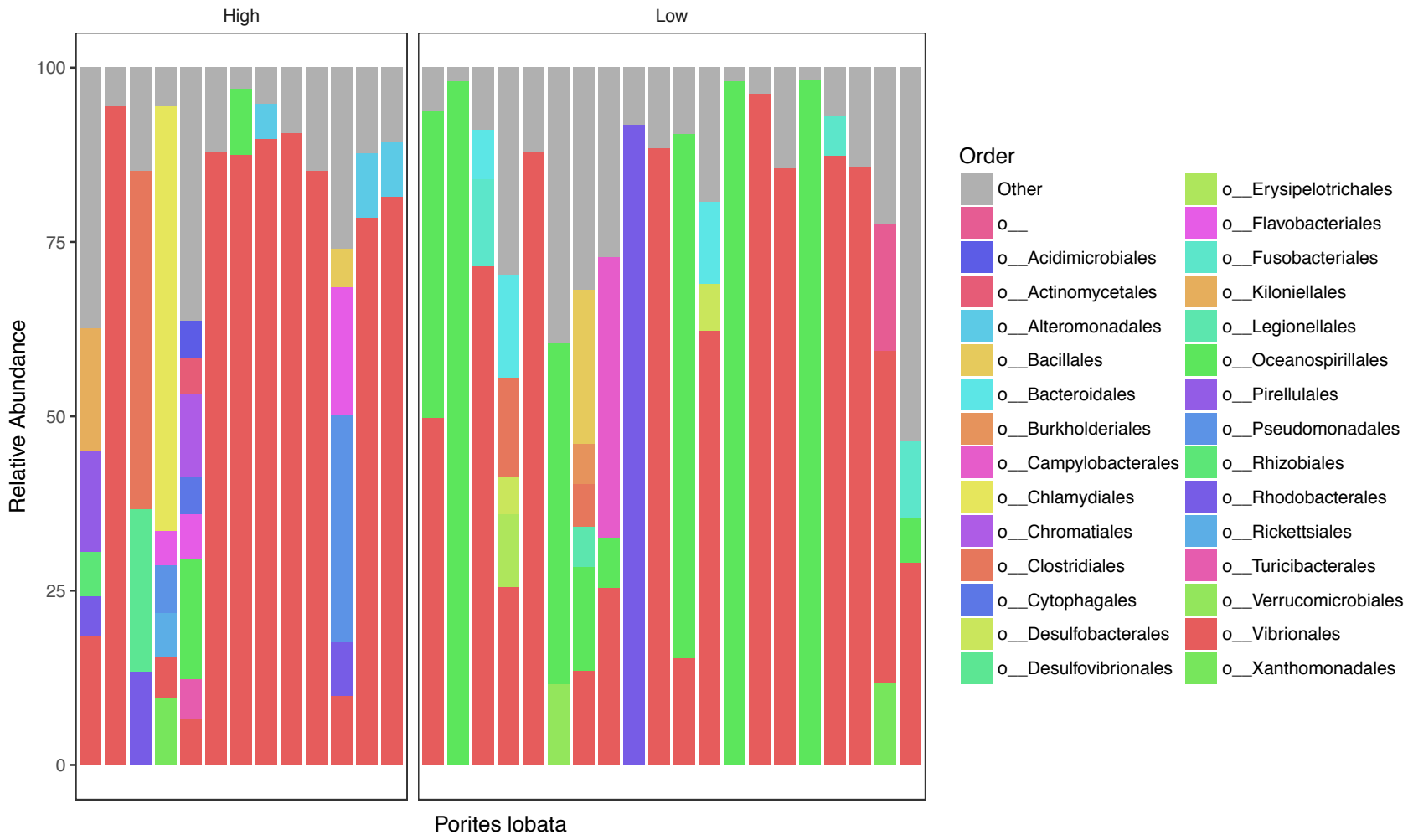

Figure B.7. Relative abundance of microbial orders for each *Porites lobata* sample during the bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any orders in less than 5% relative abundance.

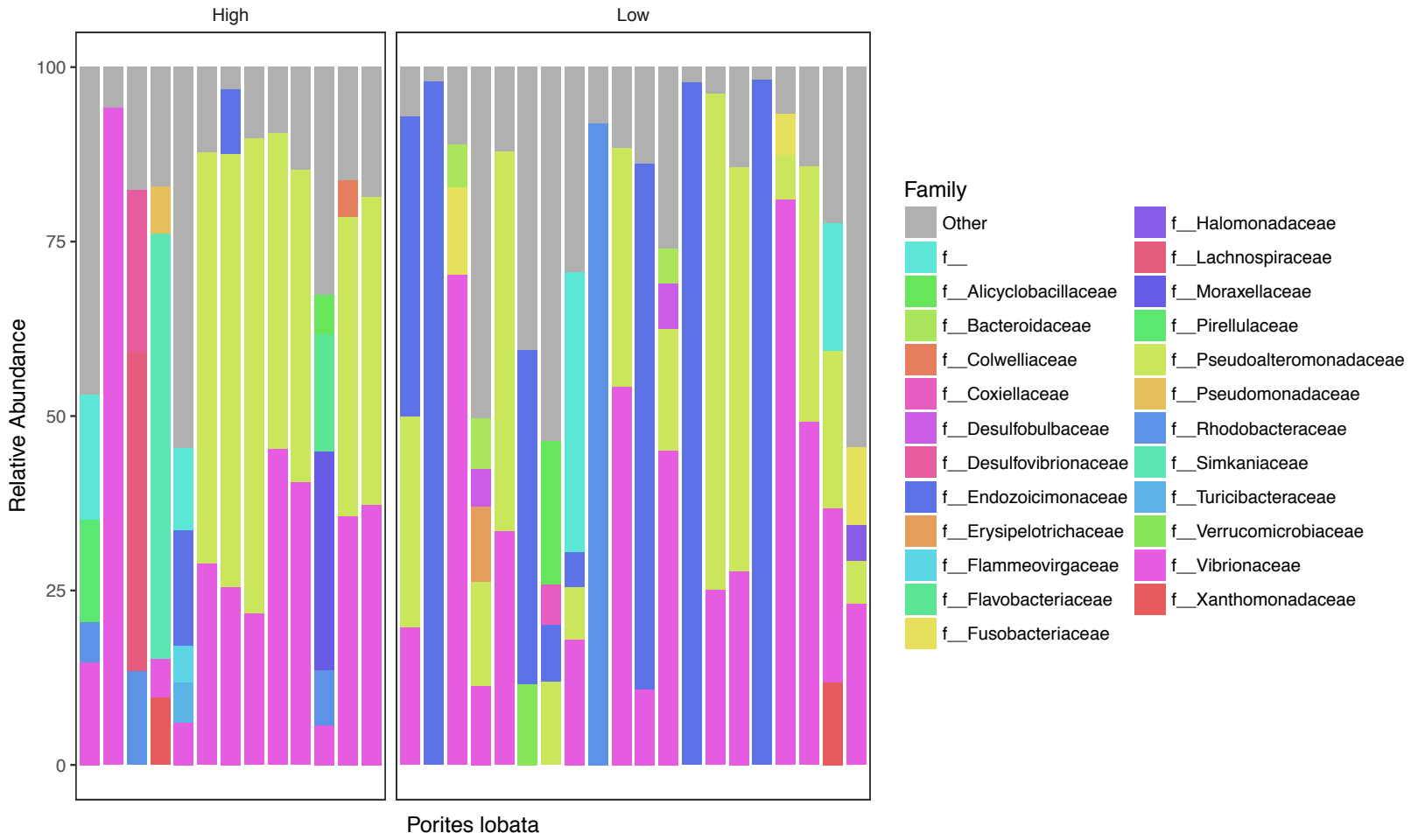

Figure B.8. Relative abundance of microbial families for each *Porites lobata* sample during the bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

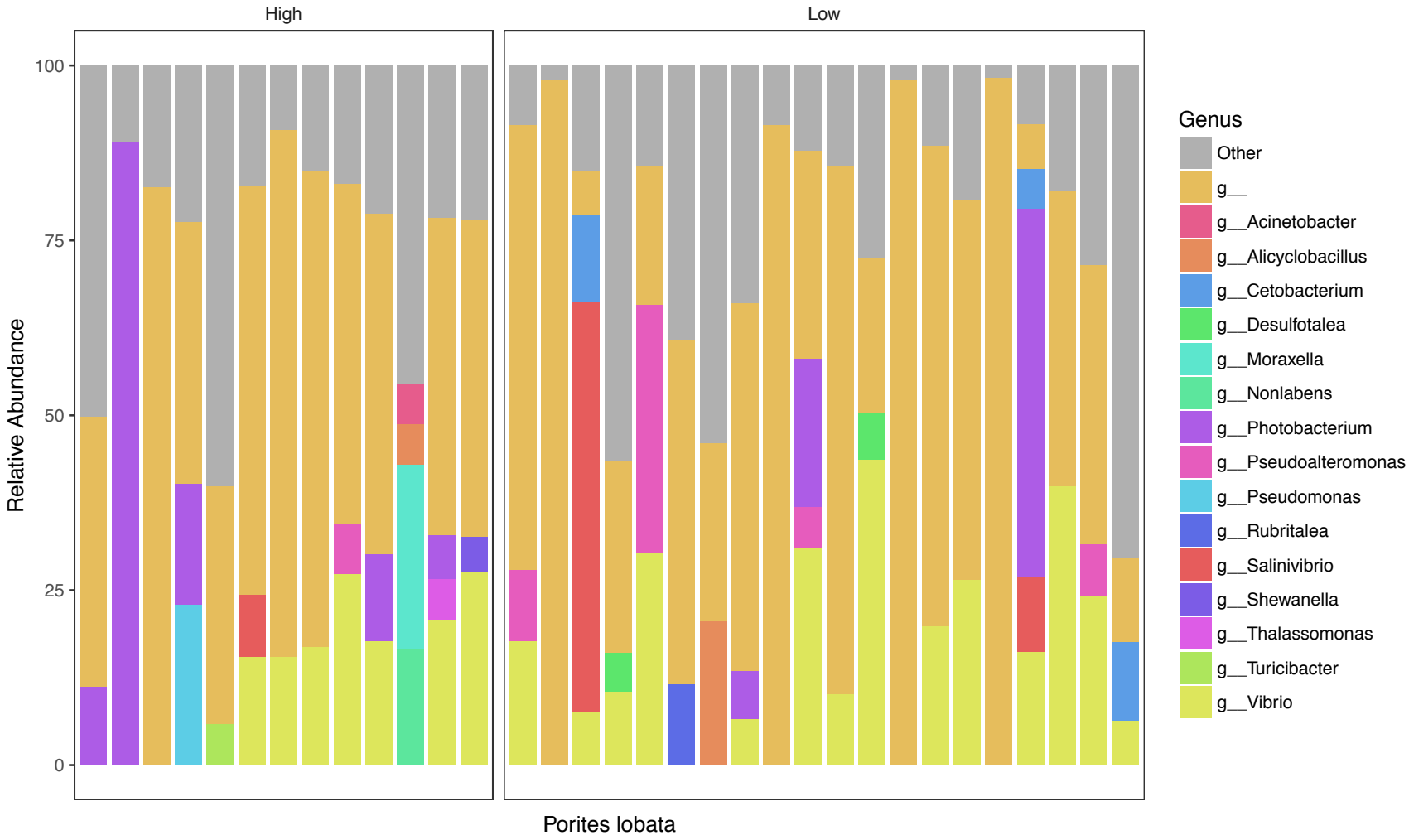

Figure B.9. Relative abundance of microbial genera for each *Porites lobata* sample during the bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

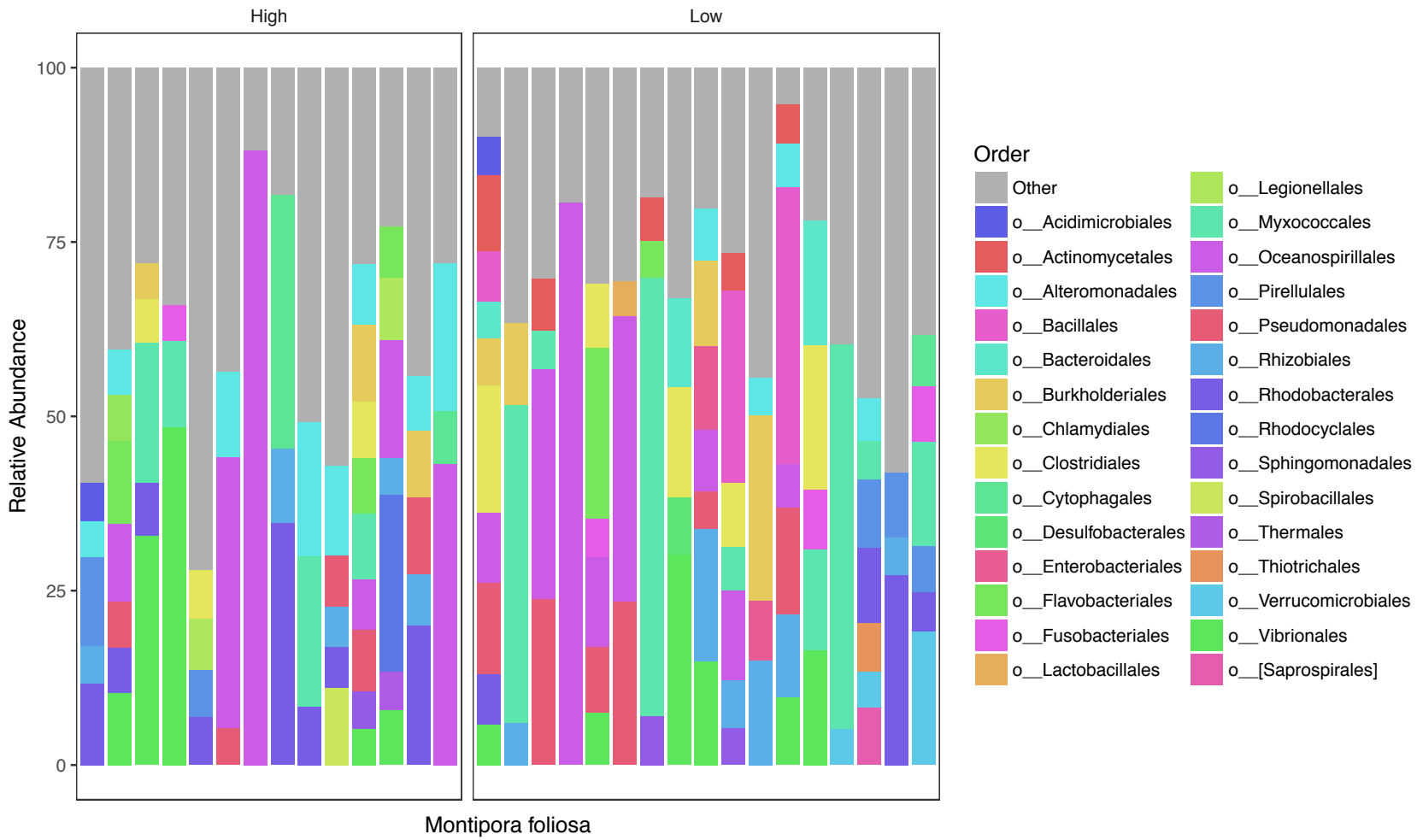

Figure B.10. Relative abundance of microbial orders for each *Montipora foliosa* sample during the bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any orders in less than 5% relative abundance.

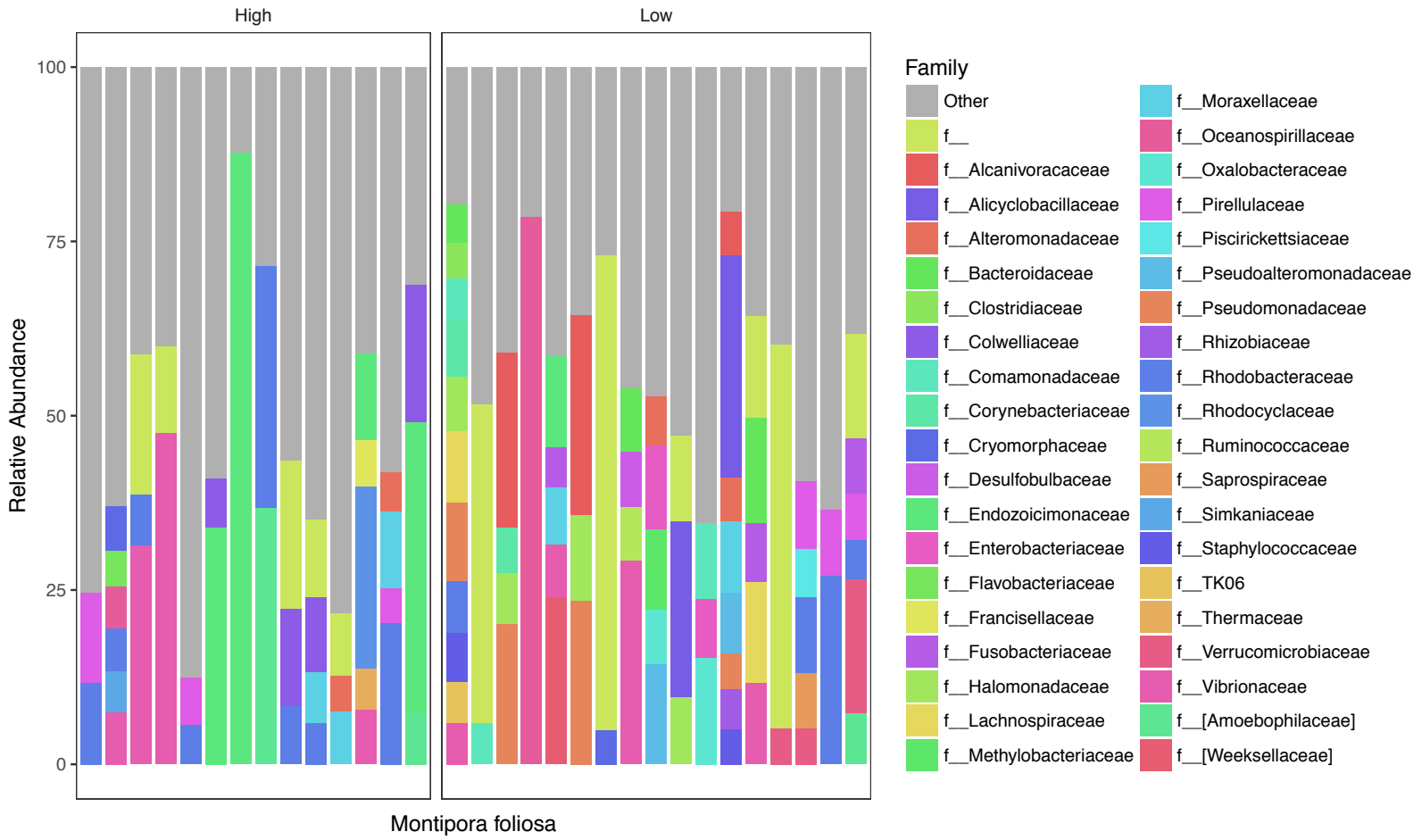

Figure B.11. Relative abundance of microbial families for each *Montipora foliosa* sample during the bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

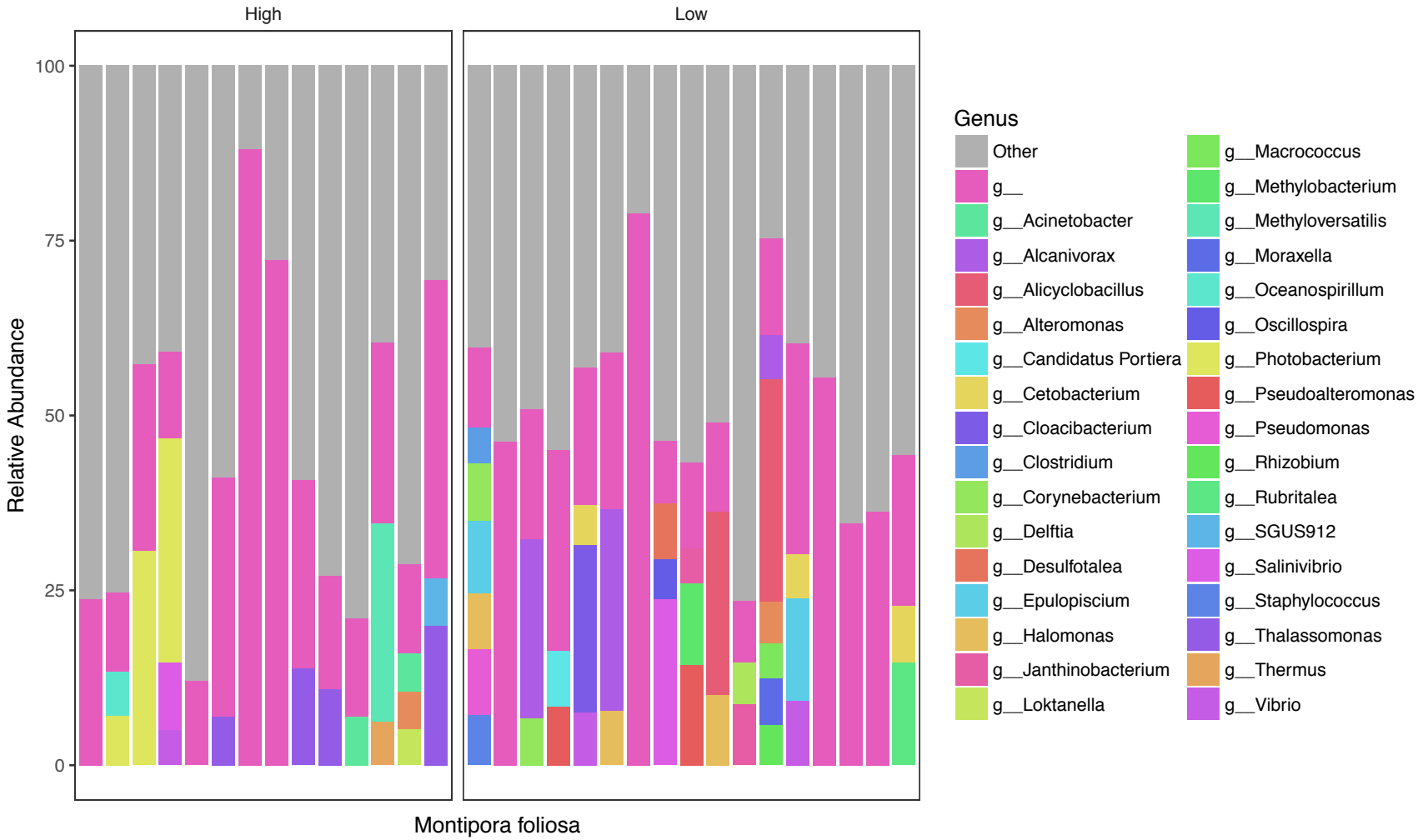

Figure B.12. Relative abundance of microbial genera for each *Montipora foliosa* sample during the bleaching hotspot in high and low human disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.