



Variable interaction outcomes of local disturbance and El Niño-induced heat stress on coral microbiome alpha and beta diversity

Jamie M. McDevitt-Irwin^{1,2} · Melissa Garren³ ·
Ryan McMinds⁴ · Rebecca Vega Thurber⁴ · Julia K. Baum^{1,5} 

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Abstract Emerging evidence indicates that individual stressors can modify the coral microbiome; however, few studies have examined the impacts of multiple stressors through natural climatic events. During periods of low and high heat stress associated with the 2015–2016 El Niño, we tracked the microbiomes of two coral species (*Porites lobata* and *Montipora aequituberculata*) across sites on Kiritimati (Christmas) Island with different levels of local disturbance (i.e. subsistence fishing, pollution, dredging). At low heat stress, local disturbance was associated with increased microbial alpha diversity (i.e. number of microbial OTUs and their relative abundance) in both species and increased beta diversity (i.e. coral to coral variation in microbial community composition) in *P. lobata*. High levels of thermal stress subsequently elevated microbial beta diversity in

both species at the low disturbance sites up to the level experienced at the high disturbance sites under low heat stress, illustrating that each stressor can destabilize the coral microbiome. However, with high heat stress microbial alpha diversity was no longer significantly different between disturbance levels for either species. Survival of *P. lobata* throughout the entire El Niño event was greater at low disturbance sites than high ones (40% vs. 15%), suggesting that protection from local stressors may enhance survival of stress-tolerant corals. However, no *M. aequituberculata* tracked in this study survived the thermal anomaly. Whether enhanced survivorship can be directly attributed to lower microbial diversity, however, remains to be tested. Overall, we found that, rather than acting synergistically, multiple stressors either acted antagonistically to one another (alpha diversity for both coral species, beta diversity for *P. lobata*) or exhibited dominance (beta diversity for *M. aequituberculata*), suggesting that multiple stressors cause various interaction outcomes on the coral microbiome and highlighting the need for future research to evaluate these interactions and their consequences for coral resilience.

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✉ Jamie M. McDevitt-Irwin
jmcirwin@stanford.edu

¹ Department of Biology, University of Victoria, 3800 Finnerty Rd, Victoria, BC V8P 5C2, Canada

² Department of Biology, Stanford University, 371 Serra Mall, Stanford, CA 94305, USA

³ School of Natural Sciences, California State University Monterey Bay, 100 Campus Center, Seaside, CA 93955, USA

⁴ Department of Microbiology, Oregon State University, 454 Nash Hall, Corvallis, OR 97331, USA

⁵ Hawai'i Institute of Marine Biology, University of Hawai'i, 46-007 Lilipuna Rd, Honolulu, HI 96744, USA

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Introduction

Of the many stressors now imposed upon the world's coral reefs, climate change-associated pulse warming events arguably pose the most imminent threat (Hoegh-Guldberg et al. 2007; Carpenter et al. 2008; De'ath et al. 2012; Ainsworth et al. 2016; Hughes et al. 2017, 2018). Recent and extreme thermal anomalies, most notably the

2015–2016 El Niño event, have heightened the need to understand the mechanisms that confer resilience to corals under elevated temperatures, including the role of the coral microbiome (Ainsworth and Gates 2016). The coral microbiome (or coral-associated microbial community) is distinct from that of the microbial communities in the surrounding reef water and sediment (McKew et al. 2012) and can also vary across compartments (i.e. tissue, skeleton, mucus and gut) of the same coral colony (Sweet et al. 2010; Blackall et al. 2015). Coral-associated microbes can play beneficial roles, including nutrient cycling (Lesser et al. 2004; Wegley et al. 2007; Raina et al. 2009; Lema et al. 2012; Ceh et al. 2013) and protection from other bacteria (Ritchie 2006; Rypien et al. 2010; Kvennefors et al. 2011; Krediet et al. 2012; Welsh et al. 2016; McDevitt-Irwin et al. 2017). Yet microbial pathogens and opportunists can also harm corals, causing tissue death through bleaching (Kushmaro et al. 1998, 2001; Ben-Haim and Rosenberg 2002) and/or disease (Sutherland et al. 2011). Maintenance of the core coral microbiome is thus considered to be essential to coral and reef health (Glasl et al. 2016).

Understanding how anthropogenic stressors alter coral microbiomes, and the broader implications for the future of coral reef health, is a crucial task for researchers (Ainsworth and Gates 2016; McDevitt-Irwin et al. 2017). Recent studies have shown that stressors can increase microbial richness (Vega Thurber et al. 2012; Santos et al. 2014; Tout et al. 2015; Zaneveld et al. 2016) and alpha diversity (Bourne et al. 2007; Meron et al. 2011; Morrow et al. 2012; Jessen et al. 2013; Röthig et al. 2016; Lee et al. 2016; Ziegler et al. 2016a) within coral colonies. Furthermore, it has been recently shown that dispersion or the increase in ‘beta diversity’ (i.e. the variation in microbiome composition from coral to coral) is a marker of stressed microbiomes (Moeller et al. 2013; Zaneveld et al. 2016, 2017).

Although coral reefs are typically subject to multiple ecosystem stressors (Ban et al. 2014), and multiple stressors can have varying interaction outcomes (i.e. synergisms, antagonisms and additive effects) (Côté et al. 2016), few studies have examined the impact of more than one stressor on the coral microbiome (McDevitt-Irwin et al. 2017). Thus, while those studies that have manipulated multiple stressors on corals have shown that these cause significant changes in the coral microbiome (Vega Thurber et al. 2009; Jessen et al. 2013; Welsh et al. 2016; Zaneveld et al. 2016; Wang et al. 2018), how non-experimental multiple stressors impact coral microbiomes in natural ecosystems remains to be seen.

Here, we capitalized on a natural factorial experiment in which heat stress from the 2015–2016 El Niño event was overlaid on two coral species at sites on Kiritimati (Christmas) atoll with different background levels of

chronic local disturbance, i.e. subsistence fishing, pollution (Walsh 2011). We tagged individual colonies of two coral species, *Porites lobata* and *Montipora aequituberculata*, at two sites with high local disturbance and two sites with low local disturbance on the atoll (Fig. 1; Watson et al. 2016). We collected tissue samples from these corals once during ‘low heat stress’ at the initiation of the 2015 El Niño event, and once 2 months later when the corals had experienced ‘high heat stress’. We then utilized 16S rRNA sequencing of these tissue samples ($n = 103$) to assess how the two stressors impacted the coral microbiome. We hypothesized that: (1) individual coral species would have distinct microbiomes, and the microbiome of each coral species would depend significantly upon the level of local disturbance, demonstrating both host specificity and the impact of local disturbance; (2) corals exposed to high local disturbance would have greater alpha and beta microbiome diversity because of decreased coral host capacity to regulate its microbial community when stressed; and (3) high heat stress would induce increases in microbiome alpha and beta diversity, with the greatest impact on corals under high local disturbance, because these corals were already under stress.

Materials and methods

Sample collection

We sampled four permanent forereef study sites on Kiritimati (Christmas Island; 01°52'N, 157°24'W), a large

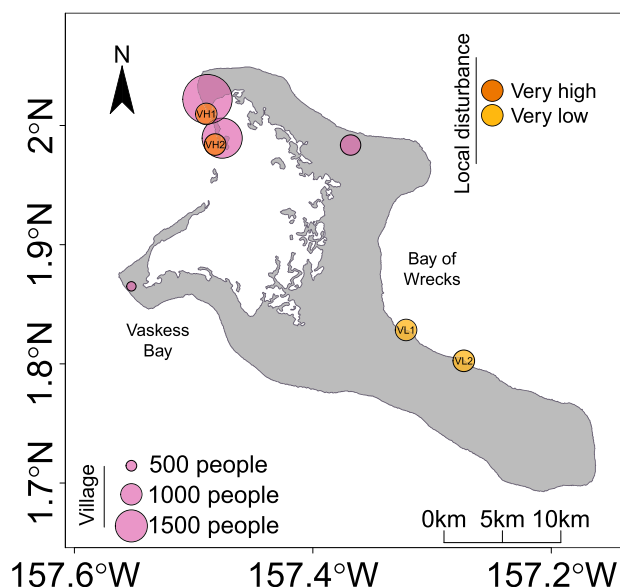


Fig. 1 Map of Kiritimati (Christmas Island) and villages with bubble size representing number of people and sampling sites designated as very low (yellow) or very high (orange) local disturbance

atoll located in the central equatorial Pacific Ocean (Fig. 1). Two of the collection sites (VH1, VH2) are exposed to a very high level of chronic local disturbance, namely subsistence fishing pressure, minor pollution from sewage run-off and dredging (at site VH1 where there is a port), due to their proximity to the atoll's main villages, Tabwakea (3001 people) and London (1895 people) (Walsh 2011; National Statistics Office 2016; Watson et al. 2016). Kiritimati does not have a sewage treatment plant, and run-off is known to lower water quality on coral reefs (Fabricius 2005; Wear and Thurber 2015). The other two collection sites (VL1, VL2) have very low levels of local disturbance, as they are not near any villages and fishing pressure (Walsh 2011; Watson et al. 2016). For simplicity, we refer to the 'very high' disturbance sites as 'high' and the 'very low' disturbance sites as 'low' throughout the manuscript.

For monitoring over the course of the 2015–2016 El Niño event, at each site, we tagged between 5 and 12 individual coral colonies of *Porites lobata* ($n = 36$ total) and *Montipora aequituberculata* ($n = 34$ total) along a 60-m transect following the 10–12 m depth isobaths. We collected tissue samples from these colonies for microbial community analysis at the start of El Niño-induced heat stress (30 April–10 May 2015) and then again during the accumulated heat stress event (2–19 July 2015). *Porites lobata* tissue samples were collected using a small chisel, while *M. aequituberculata* tissues were sampled by breaking off a small edge piece of the plating colony. Coral samples were put on ice as soon as they were brought up to the surface and then stored short term at -20°C while on island and transferred to -80°C immediately upon return to the US for storage prior to DNA extraction. In total, we collected 103 coral tissue samples, 55 from *P. lobata* and 48 from *M. aequituberculata*. Due to inclement weather, it was not possible to access all colonies during each field season. We conducted follow-up surveys in November 2016 to determine whether the coral colonies had survived the entire El Niño event, but tissue samples were not collected at this time due to high levels of coral mortality.

We also collected ancillary data to parameterize each site, including in situ water temperature and quality data, and benthic community composition data. Temperature data were recorded at 1-h intervals using Sea-Bird Temperature Loggers (SBE-56), with loggers installed at three of the sites in this study (both high disturbance sites and one low disturbance site). We collected surface water quality samples at 1-m depth at each site and immediately stored them in EPA-approved vials for nutrient analysis (i.e. nitrate plus nitrite and phosphate). To evaluate microbial community composition and abundance in the water, we collected three-to-four water samples per site approximately ~ 1 m above the benthos. Benthic

community composition at each site was analysed using 15–30 $1\text{ m} \times 1\text{ m}$ photo-quadrats taken along the 60-m transects at each time point. We estimated site- and time-specific benthic per cent cover (i.e. turf/macroalgae, CCA, and healthy coral cover), by identifying 100 randomly assigned points on each of the photographs using the program Coral Net (Beijbom et al. 2012, 2015).

Environmental sample processing

To determine the number of Degree Heating Weeks ($^{\circ}\text{C}$ -week) experienced at Kiritimati, we first defined Kiritimati's MMM as 28.14°C , using NOAA's long-term data on the Northern Line Islands (NOAA Coral Reef Watch 2013). We then applied NOAA Coral Reef Watch's DHW equation to their remote sensing temperature data (5-km resolution) (Liu et al. 2013). DHW represents the accumulation of heat stress, when sea surface temperatures are higher than the maximum monthly mean (MMM) in a region by 1°C , over the most recent 12-week period. Thus, our two sampling time points, 30 April–10 May and 2–19 July 2015, were designated as 'low heat stress' and 'high heat stress', respectively (ESM Fig. S1.1, S1.2).

We also evaluated site-specific differences in nutrients and microbial abundances in the water column. We processed three technical replicates of surface water per site for nitrate plus nitrite and phosphate concentrations at the Institute of Ocean Sciences (IOS), Sidney, BC, Canada. We preserved five millilitres of water for microbial abundance data ($n = 31$ samples, 3–4 samples per site) using a final concentration of 2% formaldehyde, while the remaining sample (750 mL–1 L) was filtered on a $0.2\text{-}\mu\text{m}$ black polycarbonate filter paper. We quantified microbial abundance using one-fourth of the filter paper on a slide stained with DAPI stain. Using a Zeiss Universal epifluorescence microscope, we took 20–31 photographs per slide using a grid search pattern to ensure no two fields of view overlapped. We counted an average of 31 cells per field of view to ensure no statistical bias from counting (Kirchman et al. 1982). The other 3/4 s of the filter was placed in RNAlater for 16S sequencing of the water community ($n = 23$), stored at -20°C while on island and then transferred to -80°C for long-term storage.

16S sequence analysis and data treatment

We extracted holobiont DNA following the Earth Microbiome Project protocol for both the reef water and coral samples (EMP 2016). We utilized 16S rRNA gene amplification of the V4 region by using the modified Earth Microbiome Project primers 515fb/806rb, a two-step polymerase chain reaction protocol, and a single Illumina

MiSeq sequence run. Sequences were processed using the Quantitative Insights into Microbial Ecology (QIIME) (version 1.9.1) (Caporaso et al. 2010) for demultiplexing, quality filtering and clustering. For detailed methods of DNA extraction, primers, PCR conditions and sequence analysis, see ESM S2 and methods for dealing with negative controls in ESM S3.

To account for differences in library size (i.e. number of reads per sample, sampling effort) between samples, we used two separate methods depending on the statistical analysis to be employed. For multivariate analyses (i.e. beta diversity, ordinations, PERMANOVA), we transformed our 16S data into relative abundances (i.e. dividing the abundance of the OTU by the overall sample sum) as it has been shown that for multivariate clustering methods with the Bray–Curtis distance, converting read abundance to proportions outperforms other normalization techniques (McMurdie and Holmes 2014). It is also becoming more common to use these proportions in microbiome studies (Hester et al. 2016; Lagkouvardos et al. 2017). However, for alpha diversity analyses, we rarefied our OTU table to the lowest read number (866) to deal with differences in library sizes.

Statistical analyses

For environmental analyses, we used linear models to assess the major drivers of bacterial abundance in a global model with sampling time point (i.e. low and high heat stresses) and disturbance level (i.e. high or low) as interacting explanatory covariates. We included site as a fixed nested effect within local disturbance; this approach can account for non-independence between sites when there are not enough levels to treat the variable as a random effect (Schielzeth and Nakagawa 2013). We used the package *lsmeans* in *R* to obtain least squares means for the best model, and post hoc contrasts to determine significant differences between disturbance levels (Lenth 2016).

We evaluated microbial alpha diversity using the Shannon Index as the response variable in models, because it is useful in situations where rare species are expected to be as important as abundant ones (Morris et al. 2014). We tested the influence of local disturbance alone first, using data from our initial sampling time point in a linear model with coral species and local disturbance (site nested as above) as fixed effects, and a two-way interaction between these variables. We then examined the influence of heat stress, using a model with the same structure but with data from the high heat stress sampling point. For each set of models, we selected the ‘best’ model according to that which had the lowest AICc (Burnham and Anderson 2002) and extracted the least squares means for the best model using the *R* package *lsmeans* (Lenth 2016).

We assessed differences in microbial beta diversity across coral colonies of the same species using the PERMDISP test with 9999 permutations on each coral species (test conducted on the relative abundance standardized OTU table). This test was conducted between local disturbance levels during both levels of heat stress. In addition, we evaluated changes in beta diversity from the low to high heat stress level within each disturbance level.

To evaluate differences in microbiome community composition, we first used unconstrained (principal coordinates analysis (PCoA)) ordinations to visualize differences between our explanatory variables and then used constrained (distance-based RDA) ordinations and PERMANOVAs (with 9999 permutations) using the Bray–Curtis dissimilarity to formally test for statistical differences between covariates. To evaluate the low and high heat stress time points separately, we conducted both a db-RDA and PERMANOVA with the starting factors coral species and local disturbance (with site nested) and used backwards stepwise ANOVAs (with 9999 permutations) to select the final model for the db-RDA.

All statistical analyses were conducted in *R* (version 3.4.1) using the *phyloseq* (McMurdie and Holmes 2013), *vegan* (Oksanen et al. 2016), *lsmeans* (Lenth 2016) and *MuMIn* (Barton 2016) packages. Code for all analyses is available on GitHub at https://github.com/baumlab/McDevittIrwin_etal_2019_CoralReefs, and raw sequence data are available at Harvard DataVerse at <https://doi.org/10.7910/DVN/3QZTT1>.

Results

Multiple stressors

Heat stress on Kiritimati increased between the two sampling periods, with average sea surface temperature rising from 28.87 °C (low heat stress) to 29.43 °C (high heat stress) according to the in situ temperature data (ESM Fig. S1.1). By the end of the second sampling period, Kiritimati’s corals had experienced 15.15 °C-week DHWs in the low disturbance level and 14.39 °C-week DHWs in the high disturbance level, placing them well above Bleaching Alert Level 2 (Liu et al. 2013) (ESM Fig. S1.2). The proportion of corals bleaching, with bleaching defined as any portion of the coral bleaching, also increased substantially from the low to high heat stress time point [*M. aequituberculata*: low heat stress (11.7%), high heat stress (51.6%); *P. lobata*: low heat stress (50%), high heat stress (93.8%)].

Differences in local disturbance levels between sampling regions were reflected in significant differences in water column microbial communities and benthic

community composition (ESM S1.3, S1.4). High disturbance sites had significantly higher microbe abundances in the water column than the low disturbance sites (t -ratio = 12.54, $df = 28$, p value < 0.0001); these differences did not change significantly as heat stress increased (Fig. 2, Table 1). Water microbial community composition also differed significantly between disturbance levels (ESM Fig. S1.3). Low disturbance sites had higher healthy coral ($55.1\% \pm 3.0$ vs. $2.3\% \pm 1.5$) and crustose coralline algae (CCA, $6.9\% \pm 0.5$ vs. $0.06\% \pm 0.1$) cover, and lower turf/macroalgal cover ($30.6\% \pm 4.0$ vs. $36.5\% \pm 24.8$) than the high disturbance level (ESM Fig. S1.4), but also higher nutrient levels in the water column (ESM Fig. S1.5).

Influence of multiple stressors on coral microbiome diversity and community composition

Microbial alpha diversity differed significantly depending on local disturbance, heat stress and coral host species. At low disturbance sites and low heat stress, *M. aequituberculata* had significantly higher alpha diversity than *P. lobata* (Table 1, Fig. 3a, t -ratio = 3.716, p value = 0.0007, $df = 36$). Local disturbance alone significantly increased microbiome alpha diversity within both coral species (Table 1, Fig. 3a; t -ratio = 4.319, p value = 0.0001, $df = 36$). High heat stress at low disturbance sites had no effect

on the microbiome of either species (Fig. 3a, b). Once under high heat stress, however, the differences in alpha diversity between local disturbance levels were lost in both coral species, because of declines in microbiome alpha diversity at the high disturbance sites (Table 1, Fig. 3b). Under high heat stress, microbiome alpha diversity of *M. aequituberculata* was still significantly higher than that of *P. lobata* (Fig. 3b, t -ratio = 4.14, p value = 0.0001, $df = 62$).

We also detected species-specific responses of coral microbiome beta diversity between disturbance levels, but unlike alpha diversity, we found that both local disturbance and heat stress increased microbial beta diversity. Under low heat stress, microbiome beta diversity amongst colonies of *P. lobata* was significantly higher at high disturbance sites than at low ones (Fig. 4a; $F = 24.72$, $p = 0.0001$, $df = 1$). Once under high heat stress, however, this difference disappeared (Fig. 3b) because of a significant increase in the microbial beta diversity amongst *P. lobata* coral colonies at the low disturbance sites (Fig. 5a; $F = 35.19$, $p = 0.0001$, $df = 1$) and the lack of change at high disturbance ones (Fig. 5b). Although the microbiome beta diversity of *M. aequituberculata* was not significantly different between disturbance levels during low heat stress (Fig. 4c), its response to high heat stress was similar to that of *P. lobata*: microbiome beta diversity increased (slightly)

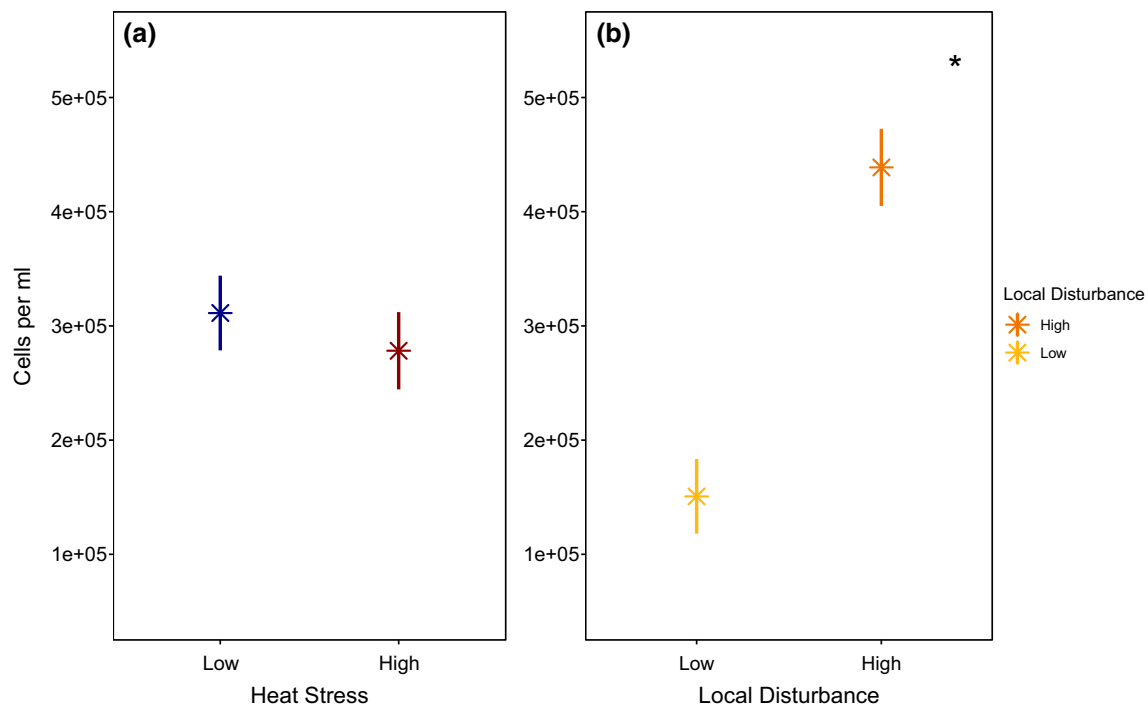
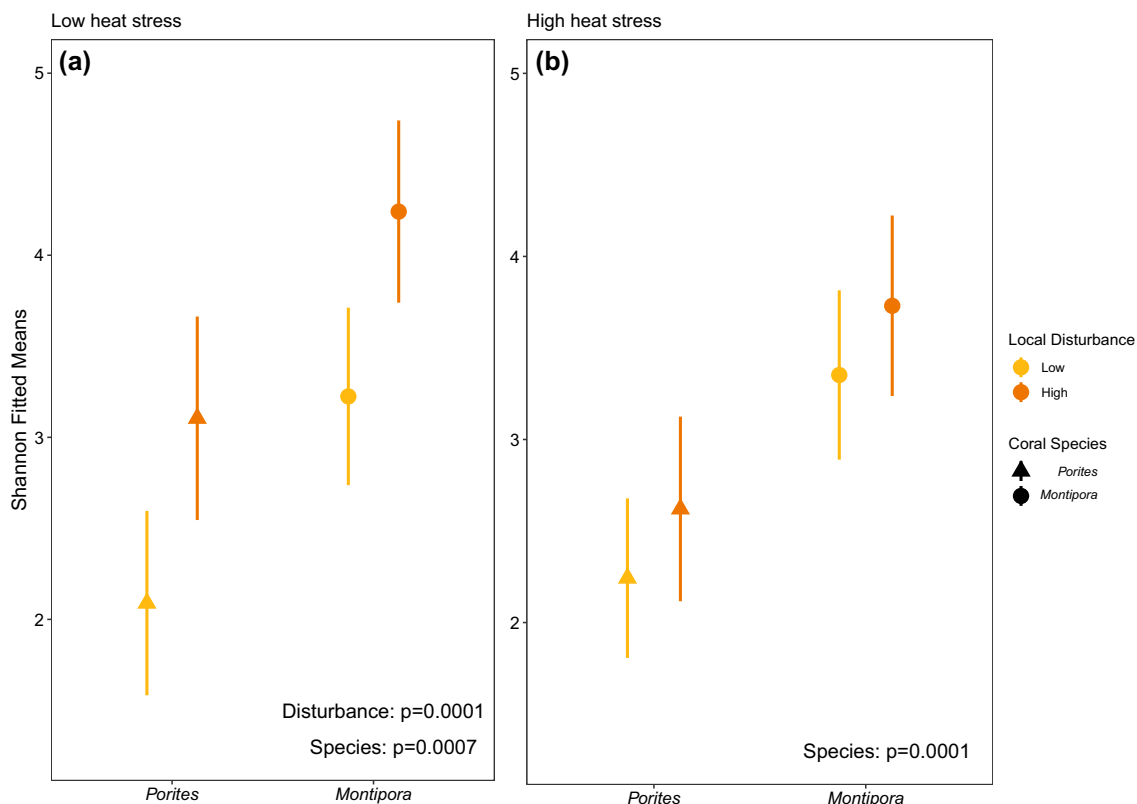


Fig. 2 Microbial abundance estimates (mean cells per ml \pm 95% CI's) from the water surrounding the sampled corals according to the best model (heat stress + local disturbance) during **a** May 2015, when heat stress was 'low' (blue) and July 2015, when heat stress was

'high' (red), **b** for individual sites in the two local disturbance levels (low = yellow, high = orange), asterisk indicates significant differences from lsmeans post hoc contrasts from the best model using 0.05 as the alpha level

Table 1 Model data, explanatory variables, adjusted R^2 , AICc, delta AICc and the next best model for each of the best models after model selection for microbial abundance (environmental data) and microbial alpha diversity

Data	Best model	Adj. R^2	AICc	Δ AICc	Next best model
Microbial abundance	Heat stress + local disturbance/site	0.91	767.29	1.34	Local disturbance/site
Alpha diversity (low heat stress)	Coral species + local disturbance/site	0.45	109.56	3.53	Coral species \times local disturbance/site
Alpha diversity (high heat stress)	Coral species	0.20	197.41	4.35	Coral species + local disturbance/site

**Fig. 3** Coral-associated microbial alpha diversity (mean \pm 95% CI) between low (yellow) and high (orange) local disturbance levels for *Porites lobata* (triangles) and *Montipora aequituberculata* (circles)

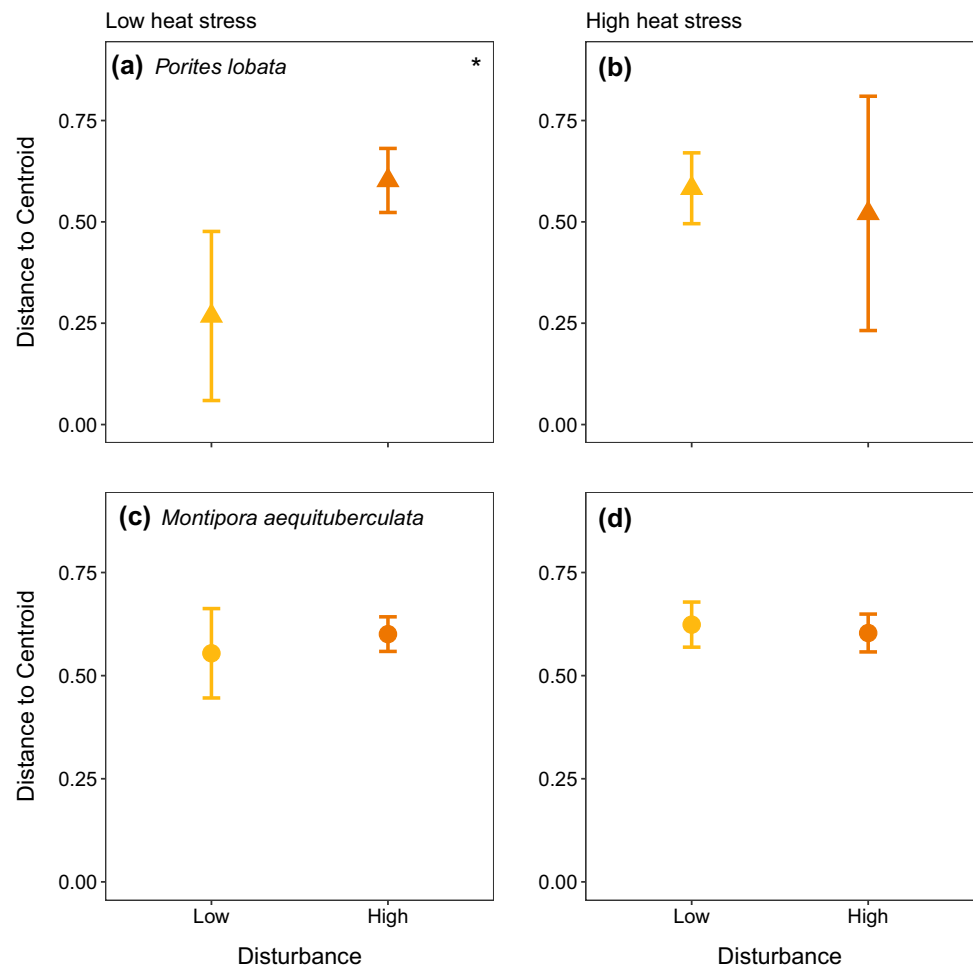
at the low disturbance level (Fig. 5c; $F = 4.45$, $p = 0.0478$, $df = 1$), and showed no change at the high disturbance level (Fig. 5d).

Initially, coral microbiome community composition differed significantly between disturbance levels (PERMANOVA, Pseudo- $F = 3.69$, $p = 0.0007$, $df = 1$) and coral species (PERMANOVA, Pseudo- $F = 7.27$, $p = 0.0001$, $df = 1$) (Fig. 6a, ESM Fig. S1.6a). The *P. lobata* microbiome community was dominated by the families Pseudoalteromonadaceae and Vibrionaceae; these taxa were especially abundant within corals at the low disturbance sites (49% and 38% relative abundance, respectively), compared to the high disturbance sites (11% and 17%) where composition was more variable (ESM

during low (a), high (b) heat stress. The p values are from the lsmeans post hoc contrasts of each of the factors in the best model using 0.05 as the alpha level

Fig. S1.7, S1.8). Although *M. aequituberculata* communities also differed significantly between disturbance levels, the difference was less pronounced than in *P. lobata*. As such, under low heat stress, *M. aequituberculata* harboured fairly high proportions of the same microbial family Rhodobacteraceae, within both low and high local disturbance (12% and 13%), and most of the differences in microbial composition stemmed from the less abundant microbes (ESM Fig. S1.9, S1.10). Microbial community differences between the two species (PERMANOVA, $F = 6.94$, $p = 0.0001$, $df = 1$) and between disturbance levels (PERMANOVA, $F = 2.39$, $p = 0.0019$, $df = 1$) persisted once under high heat stress, but these factors explained less variation in the db-RDA, 27.55% versus

Fig. 4 Coral microbiome beta diversity (mean \pm SD) across coral colonies of the same species, between low (yellow) and high (orange) local disturbance levels for **a**, **b** *Porites lobata*, **c**, **d** *Montipora aequituberculata*, during low (**a**, **c**) and high heat stress (**b**, **d**) (asterisk indicates significant differences using the PERMADISP test and 0.05 as the alpha level)



18.48% (Fig. 6b, ESM S1.6b). Once under high heat stress, *P. lobata* colonies had lower relative abundance of Pseudoalteromonadaceae and higher relative abundance of Endozoicimonaceae, which increased to > 90% relative abundance in three coral colonies under low disturbance (Fig. S1.11). Under high heat stress, *M. aequituberculata* had lower relative abundance of Rhodobacteraceae in both low and high disturbance levels (4% and 8%) (ESM Fig. S1.13).

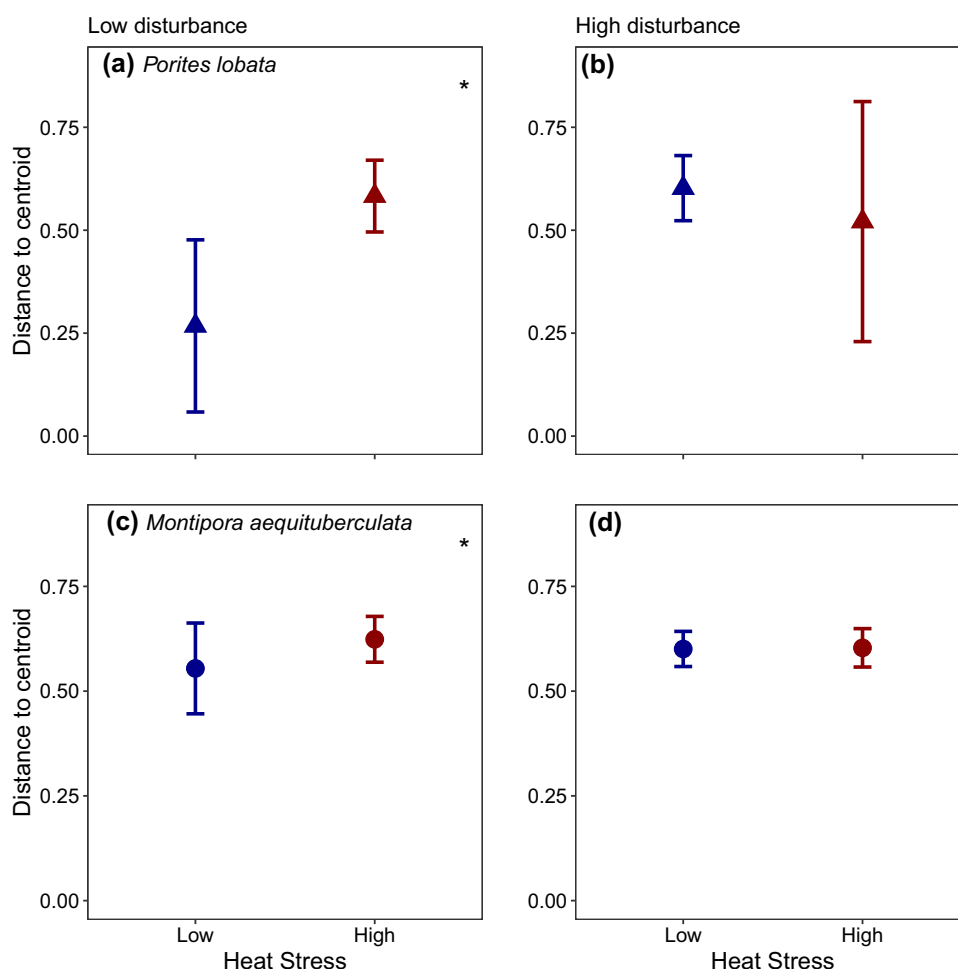
Coral survival

Amongst these tagged coral colonies, *Porites lobata* were the only survivors of the El Niño event; all tagged *M. aequituberculata* colonies died. Furthermore, *P. lobata* colonies within the low disturbance level had a higher survival rate (40%) than the colonies in the high disturbance level (15%).

Discussion

Overall, coral microbiome diversity and composition varied with host species, local disturbance and heat stress, but not always as predicted. We found support for our first hypothesis, with corals exhibiting species-specific microbiomes and clear differences between local disturbance levels. We also found support for our second hypothesis, with corals under high levels of local disturbance having higher alpha and beta microbial diversity, likely due to stressed coral hosts losing their capacity to regulate their microbiomes. However, our third hypothesis was not supported: alpha diversity did not increase with high heat stress, but rather declined at high disturbance sites, homogenizing the differences that had previously been apparent between disturbance levels. High heat stress did increase microbiome beta diversity, but only at low disturbance sites. From a multiple stressors perspective (Darling and Côté 2008; Côté et al. 2016), we found no evidence of the combined stressors acting synergistically. Rather they acted antagonistically on alpha diversity (both species) and beta diversity in *Porites lobata*; for beta

Fig. 5 Coral microbiome beta diversity (mean \pm SD) across coral colonies of the same species, between low (blue) and high (red) heat stress for **a**, **b** *Porites lobata*, **c**, **d** *Montipora aequituberculata* in **a**, **c** high, **b**, **d** low local disturbance (asterisk indicates significant differences using the PERMADISP test and 0.05 as the alpha level)



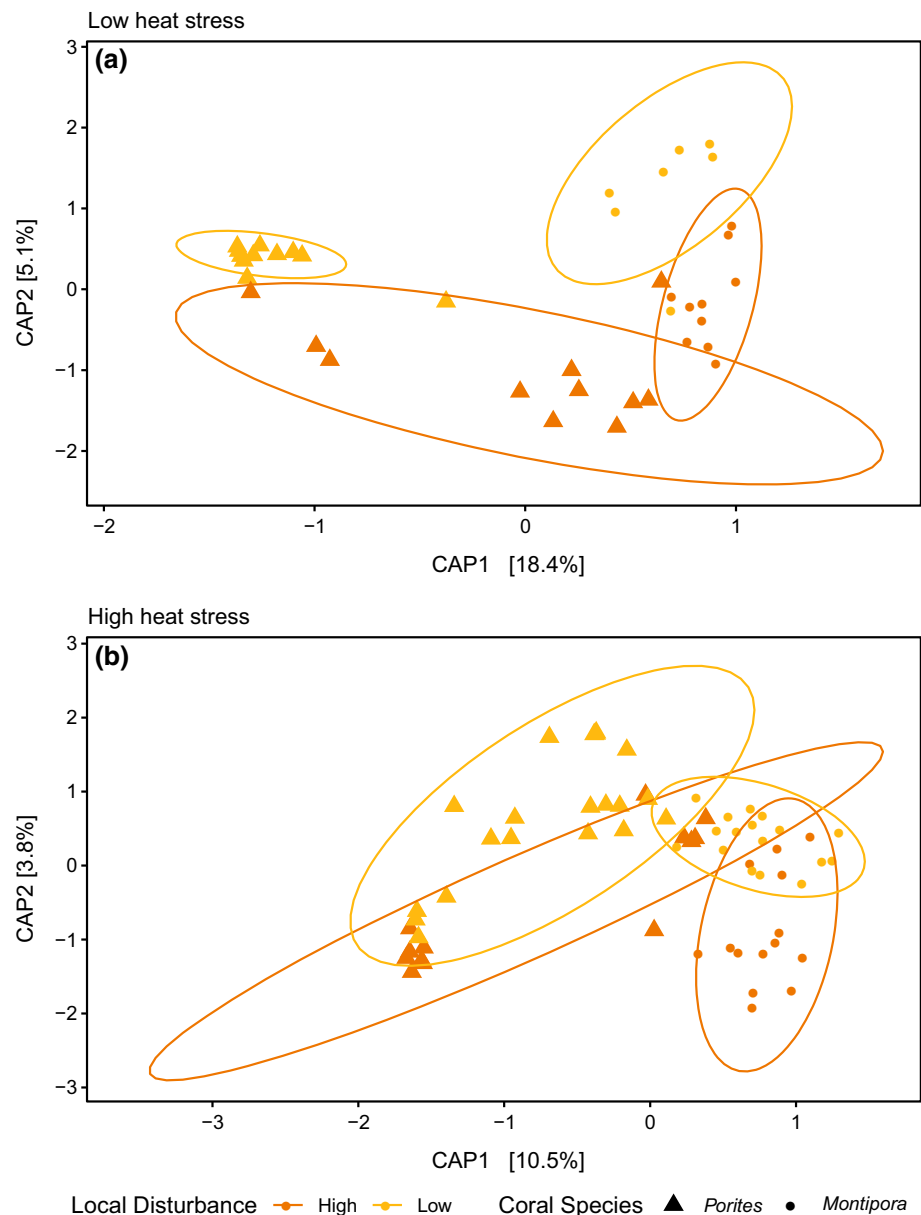
diversity in *M. aequituberculata* high heat stress outweighed the influence of local disturbance, demonstrating a dominance effect (Fig. 7). Finally, of our two species, only tagged colonies of *P. lobata* survived the 2015–2016 El Niño event. Survival was greater in the low disturbance sites (40% vs. 15% in the high disturbance level), which may be related to their initially low alpha and beta microbial diversity, but also could depend on other components of the coral (e.g. *Symbiodinium*, energy reserves, gene expression, etc.).

Multiple stressors change the environment

Each of the environmental differences detected between the sites with low and high local disturbance has the potential to influence the coral microbiome. Higher microbial abundances in the water column at sites with high local disturbance (fishing, sewage influx) provide a greater pool of microbes to invade corals; increased microbial abundance in the water column has previously been shown for a different anthropogenic disturbance, fish farms (Garren et al. 2009). Differences in water microbial

community composition may be partially explained by differences in benthic cover at each disturbance level: high disturbance sites had lower coral cover and higher turf/macroalgae cover than the low disturbance sites, and turf/macroalgae produce different dissolved organic matter than corals, selecting for lower bacterial diversity and increased virulence factors (Nelson et al. 2013). During thermal stress events, contact with turf algae also can induce shifts in the coral microbiome towards the turf microbiome (Pratte et al. 2017). In addition, there were higher nutrient levels at the low than the high disturbance sites, which can induce changes in the coral microbiome (Vega Thurber et al. 2009, 2013). Generally, high nutrients are associated with local disturbance; however, in this case, we speculate that high levels in the low disturbance sites might reflect corals in the low disturbance level recycling nutrients in the water column to create a ‘biological hot-spot’ of nutrients (Gove et al. 2016) or greater levels of coral mucus shedding, which is another means for preventing nutrient loss on coral reefs (Wild et al. 2004). Additionally, the high abundance of bacterial cells at the high disturbance sites could be sequestering nutrients, thus

Fig. 6 Microbial communities of *Porites lobata* (triangles) and *Montipora aequituberculata* (circles): **a** at sites with high (orange) and low (yellow) local disturbance under low heat stress, as illustrated by a distance-based RDA (db-RDA) using Bray–Curtis distance (best model: coral species + local disturbance/site; 27.55% of variation explained), **b** low and high local disturbance under high heat stress, as illustrated by a db-RDA using Bray–Curtis distance (best model: coral species + local disturbance/site; 18.48% of the variation explained). The ellipses are 95% confidence groupings



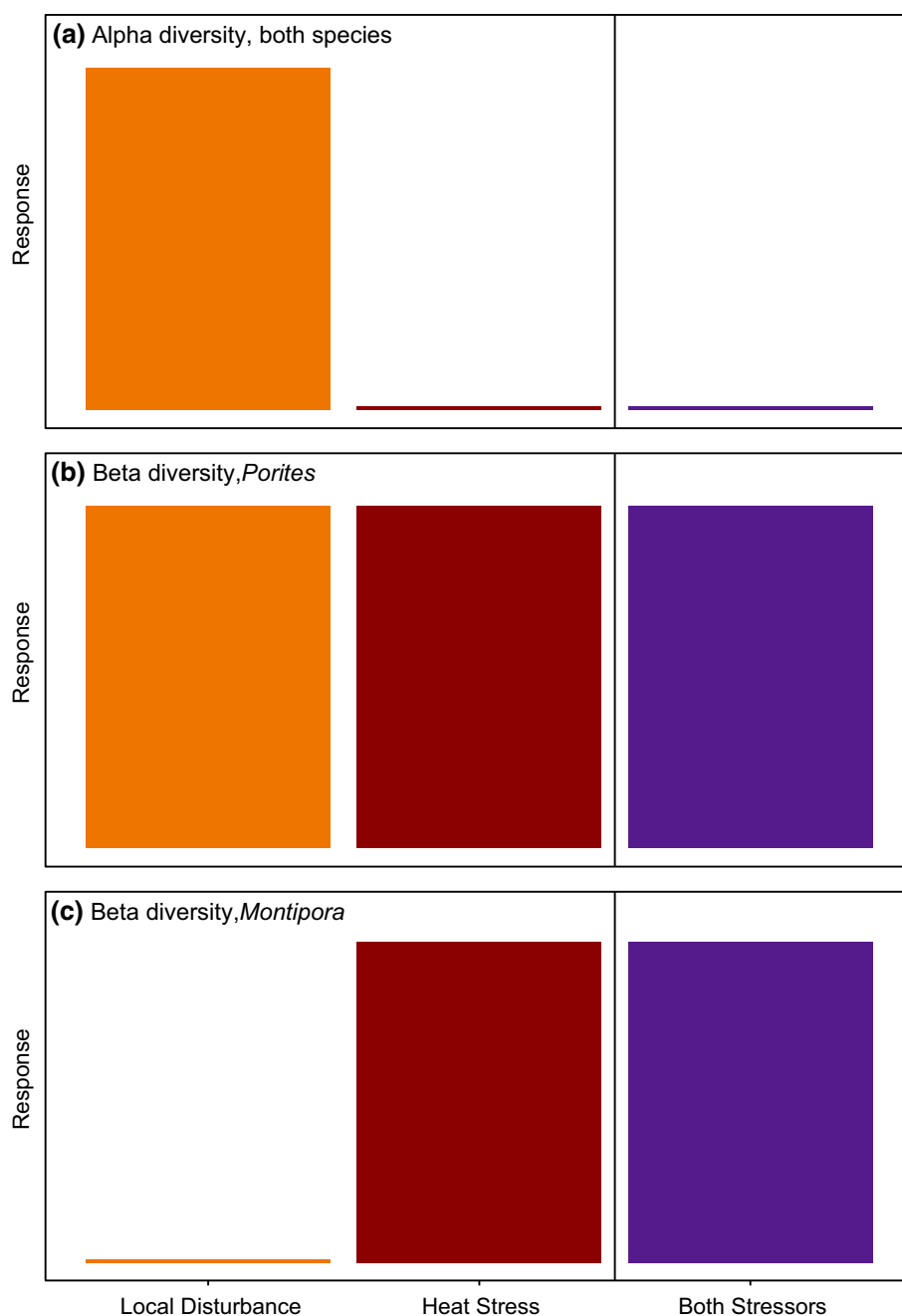
amplifying the difference in nutrient levels between the two disturbance levels. These results demonstrate the complexity of coral reef nutrient cycling and suggest the potential involvement of other factors, beyond corals, that may influence nutrients, such as ocean currents, seasonality and algae cover.

Multiple stressors have antagonistic effects on microbial alpha diversity

Local disturbance significantly increased coral microbial alpha diversity, but this effect attenuated with greater heat stress (Fig. 7a). Coral microbiome diversity has previously been shown to increase when subjected to a range of single

stressors, including elevated water temperatures (Lee et al. 2016), ocean acidification (Meron et al. 2011), water pollution (Ziegler et al. 2016b) and within diseased corals (Sunagawa et al. 2009), implying that increased microbial diversity is indicative of stressed corals that are unable to regulate their microbial community (McDevitt-Irwin et al. 2017). In accordance with these studies, we found that when subjected to local disturbance (and only minimal heat stress), Kiritimati's corals had greater microbial alpha diversity. Roder et al. (2015) also found that sites with high coral cover had lower microbiome alpha diversity and suggested that these areas where corals are abundant are optimal habitats, which may structure the coral microbiome. However, we found no evidence that the coral

Fig. 7 Conceptual figure (after Côté et al. 2016) showing the microbial community responses to the individual stressors (local disturbance, heat stress) and to the interaction between the two stressors, for each diversity metric: **a** an antagonistic interaction for the alpha diversity of both coral species, **b** an antagonistic interaction for *Porites lobata* beta diversity, **c** a dominance interaction for *Montipora aequituberculata* beta diversity (local disturbance = orange, heat stress = red, both stressors = purple)



microbiome alpha diversity of either species responded to heat stress alone, and when subjected to both local disturbance and high heat stress, alpha diversity levels declined back to the levels seen absent either stressor, indicative of an antagonistic interaction between the stressors (Côté et al. 2016). It is difficult to explain this counterintuitive finding, but with few other studies to date examining the impact of multiple stressors on the coral microbiome much remains to be learned about the nature of such interactions. One potential explanation is that heat stress has a higher selective pressure than local disturbance,

since heat stress has a more direct impact upon the microbiome. These results do suggest that changes in the alpha diversity of microbial communities should be interpreted cautiously as this may be a poor metric of stress.

Local disturbance and high heat stress increase microbial beta diversity

Unlike alpha diversity, we found that both local disturbance and high heat stress increased microbial beta diversity, albeit with species-specific responses (Fig. 7b, c). In

contrast to macroecological systems, where decreased beta diversity across sites (i.e. biotic homogenization) is a sign of a stressed ecosystem (Olden and Rooney 2006; Qian and Ricklefs 2006; Iacarella et al. 2018), recent microbial studies in systems as varied as corals (Zaneveld et al. 2016; Wang et al. 2018), the human lung (Charlson et al. 2012) and chimpanzees (Moeller et al. 2013) have shown that stressors increase microbiome beta diversity. This likely occurs because stress diminishes the host's capacity to regulate its microbial community and the community has become destabilized (Zaneveld et al. 2017). Increased microbial beta diversity in our system may reflect suppression of the coral immune pathway by coral pathogens (e.g. *Vibrio coralliilyticus* (Vidal-Dupiol et al. 2014)), heat stress (Vidal-Dupiol et al. 2014), or coral bleaching (Pinzón et al. 2015), resulting in increased microbiome variation amongst colonies of the same coral species. For *P. lobata*, both local disturbance and high heat stress increased microbial beta diversity. When the stressors were combined, however, microbial beta diversity did not increase further (i.e. either additively or synergistically), indicating an antagonistic interaction (Fig. 7b; Côté et al. 2016) and suggesting that beta diversity was already maximized by either stressor alone. Although *M. aequituberculata* microbiome beta diversity did not respond to local disturbance, it did increase significantly with high heat stress at low disturbance sites, indicating that heat stress was the dominant stressor (Fig. 7c; Côté et al. 2016). These results suggest that corals in the low disturbance area were better able to regulate their communities prior to this high heat stress.

Overall coral microbiome composition converges with multiple stressors

The community composition of coral microbiomes differed significantly between coral host species and local disturbance, but with the added stressor of high heat stress, these differences attenuated. At low heat stress, the coral microbiome of each species had more distinct clustering (i.e. more similar microbiome composition) in the low than the high disturbance level. Lee et al. (2012) found a similar pattern, with more separation in microbiome composition between coral species in a 'pristine' site than an impacted one, suggesting that corals are more selective of their microbiome on 'pristine' reefs. Under high heat stress, the microbiomes were still significantly different between coral host species and disturbance levels, but these two factors now explained less of the variation, suggesting that coral microbiome composition had become more similar. Stressors including sedimentation, sewage discharge (Ziegler et al. 2016b) and disease (Frias-Lopez et al. 2004; Roder et al. 2014) can decrease coral host specificity and

increase the similarity of microbiome composition between coral hosts, likely due to corals responding in the same manner to a stressful event. Similarly, we suggest that this increase in compositional similarity between local disturbance levels and coral species, which may initially seem contrary to the observed increase in within-species microbial beta diversity that occurred with high heat stress, indicates that the microbial beta diversity increases at the low disturbance level were driven by invading microbes that were already found within corals at the high disturbance level, thereby making the overall community composition more similar.

Although the two coral species shifted to having more similar microbiome compositions under high heat stress, each species had distinct responses to the two stressors, in terms of changes in relative abundance of their most common bacterial taxa. Pseudoalteromonadaceae and Vibrionaceae were the most common families within *Porites lobata* regardless of heat stress level, but levels of these taxa were particularly high during low heat stress at the low disturbance sites. Under these conditions, all *P. lobata* colonies were dominated by Pseudoalteromonadaceae and Vibrionaceae, suggesting microbiome stability or structuring. Vibrionaceae includes potentially pathogenic bacteria (Kushmaro et al. 2001; Mouchka et al. 2010; McDevitt-Irwin et al. 2017), while Pseudoalteromonadaceae includes potentially beneficial microbial taxa (Shnit-Orland et al. 2012). The family Pseudoalteromonadaceae, and genus *Pseudoaltermonas*, were both found at higher relative abundance within *P. lobata* at low disturbance sites, potentially providing protection through their antibacterial activity (Shnit-Orland et al. 2012). Relative abundance of Pseudoalteromonadaceae diminished in *P. lobata* in the low disturbance area once under high heat stress, suggesting a loss of colony capacity to ward off microbial invaders via its antibacterial activity (Shnit-Orland et al. 2012). Additionally, three colonies of *P. lobata* in the low disturbance level had striking increases in relative abundance of Endozoicimonaceae, which has been negatively correlated with bleaching pathogens (Pantos et al. 2015), suggesting that it may play a protective role in corals. Furthermore, Endozoicimonaceae-related bacteria tend to consistently decrease across a wide range of stressors (McDevitt-Irwin et al. 2017) including ocean acidification (Morrow et al. 2015; Webster et al. 2016) and local human impacts (Ziegler et al. 2016a). In addition, recent genomic evidence suggests that *Endozoicomonas* bacteria cycle carbohydrates and providing proteins to their hosts (Neave et al. 2017). Under low heat stress, in both local disturbance levels, *M. aequituberculata* was composed of Rhodobacteraceae, potentially opportunistic taxa that have been found on both healthy and diseased corals (Meron et al. 2011; Sharp et al. 2012) and may increase in

abundance after coral pathogens open niche space (Welsh et al. 2015). Yet during high heat stress, the relative abundance of these Rhodobacteraceae decreased within both disturbance levels.

Survival of protected stress-tolerant corals

Overall, only *P. lobata*, a coral species that is considered to have a ‘stress-tolerant’ life-history strategy (Darling et al. 2012), survived the 2015–2016 El Niño event; all *M. aequituberculata* colonies, a generalist or competitive life-history strategy (Darling et al. 2012), died. *Porites lobata* colony survival was higher within the low than the high disturbance level, suggesting that protection from local disturbances may help stress-tolerant coral species like *P. lobata* persist and survive through intense thermal stress.

It has been previously shown that local anthropogenic stressors can decrease coral resilience to global stressors, by decreasing coral growth rates after a major bleaching event (Carilli et al. 2009). It remains to be seen to what extent higher survival is related to microbial communities, such as *P. lobata*’s initially lower alpha and beta microbial diversity, or to biological components of the coral colony (e.g. *Symbiodinium*, energy reserves, gene expression, etc.).

In conclusion, our study provides evidence that both local disturbance and high heat stress can destabilize the coral microbiome via significant changes to microbiome alpha and beta diversity, and concomitant changes in underlying microbial community composition. Increases in microbiome alpha (to local disturbance) and beta diversity (to local disturbance and high heat stress) may be due to stressed coral colonies being unable to regulate incoming microbes (Zaneveld et al. 2017). Interactions between the two stressors were, however, somewhat unanticipated, whereas additive or synergistic effects of multiple stressors are of greatest conservation concern (Côté et al. 2016); here, instead we found evidence of antagonistic effects, in which the combined effect of both stressors was either less than (alpha diversity for both species) or equal to (beta diversity for *P. lobata*) the effect of the single stressors, and dominance effects (beta diversity for *M. aequituberculata*), in which the combined effect of both stressors equalled that of the dominant stressor, high heat stress. Finally, our results demonstrate that stress-tolerant corals like *P. lobata* can survive intense El Niño events, especially if protected from local disturbance. Future studies should build from these results to further investigate the impacts of multiple stressors on the coral microbiome and the implications of changes in microbiome diversity and composition for coral resilience.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states there is no conflict of interest.

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Electronic Supplementary Material S1

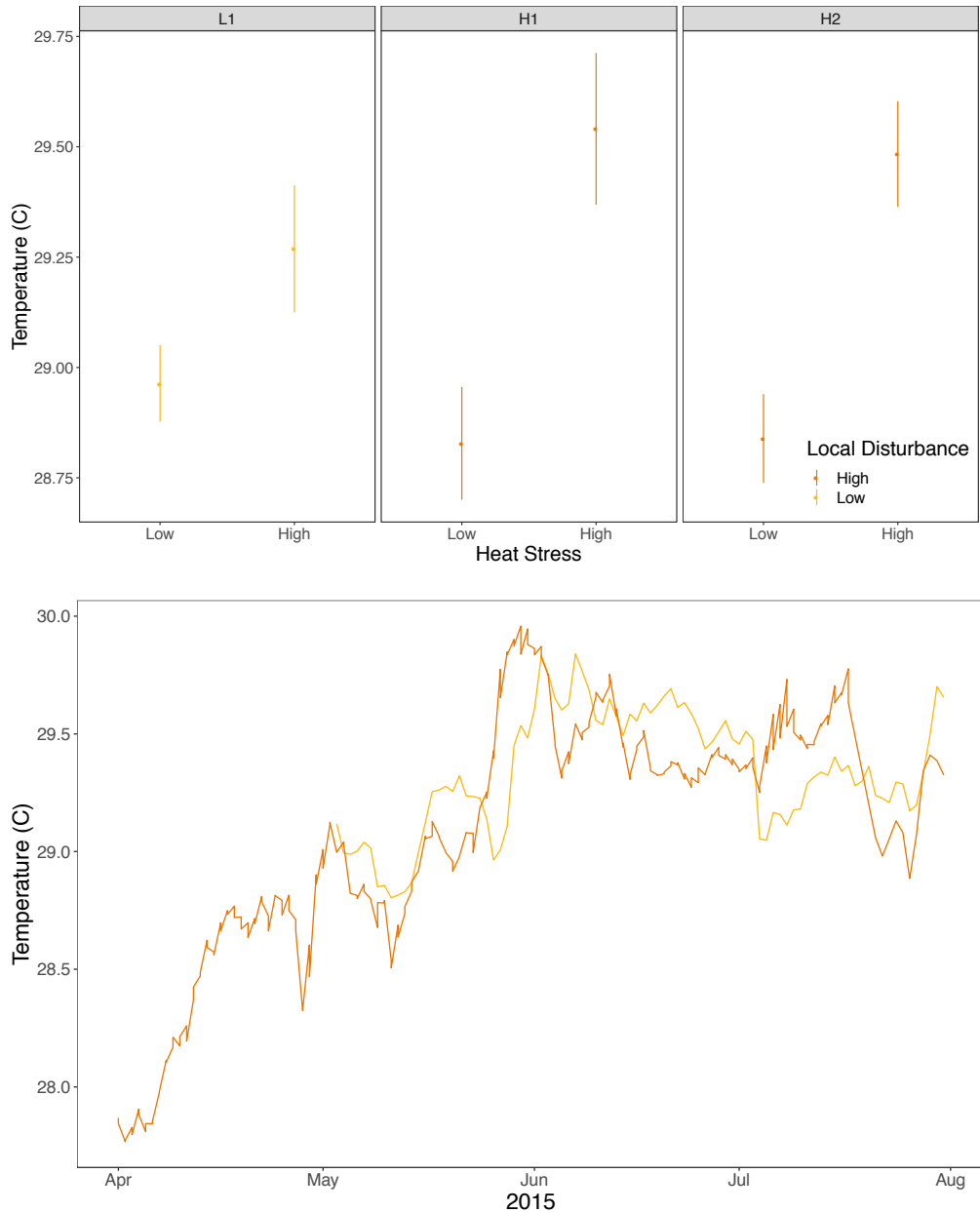


Figure S1.1. Seawater temperature on Kiritimati at each of three sites at which Seabird SBE56 temperature loggers were deployed (high disturbance=orange; low disturbance=yellow), color coded by disturbance level, and shown for the low and high heat stress periods (top). Overall temperature for the high and low disturbance levels from April 2015 to August 2015 (bottom) (low heat stress sampling period= April 30-May 10; high heat stress sampling period=July 2-19). Temperature is plotted by site to demonstrate any site variation.

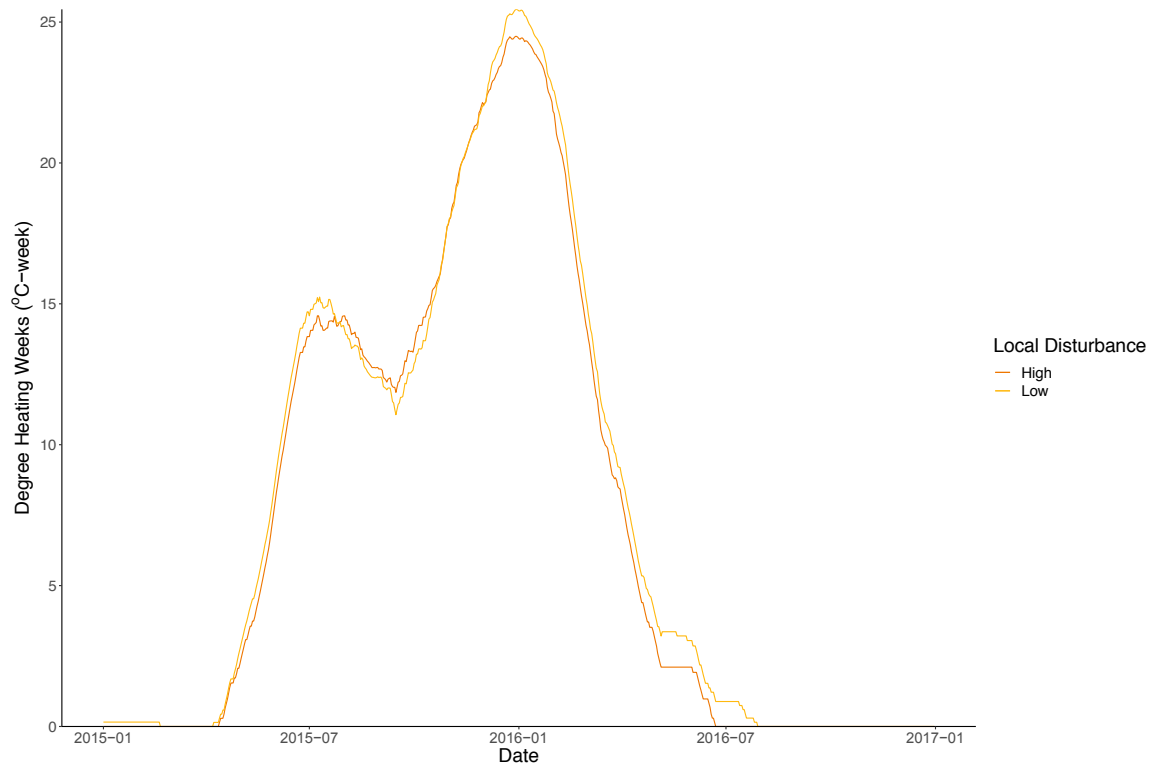


Figure S1.2. Degree heating weeks (°C-week) at Kiritimati from January 2015-April 2016 from NOAA DHW remote sensing data (5km- resolution) for both the low (yellow) and high (orange) local disturbance level. The grey shading indicates the two sampling time points, the first during low heat stress and the second during high heat stress.

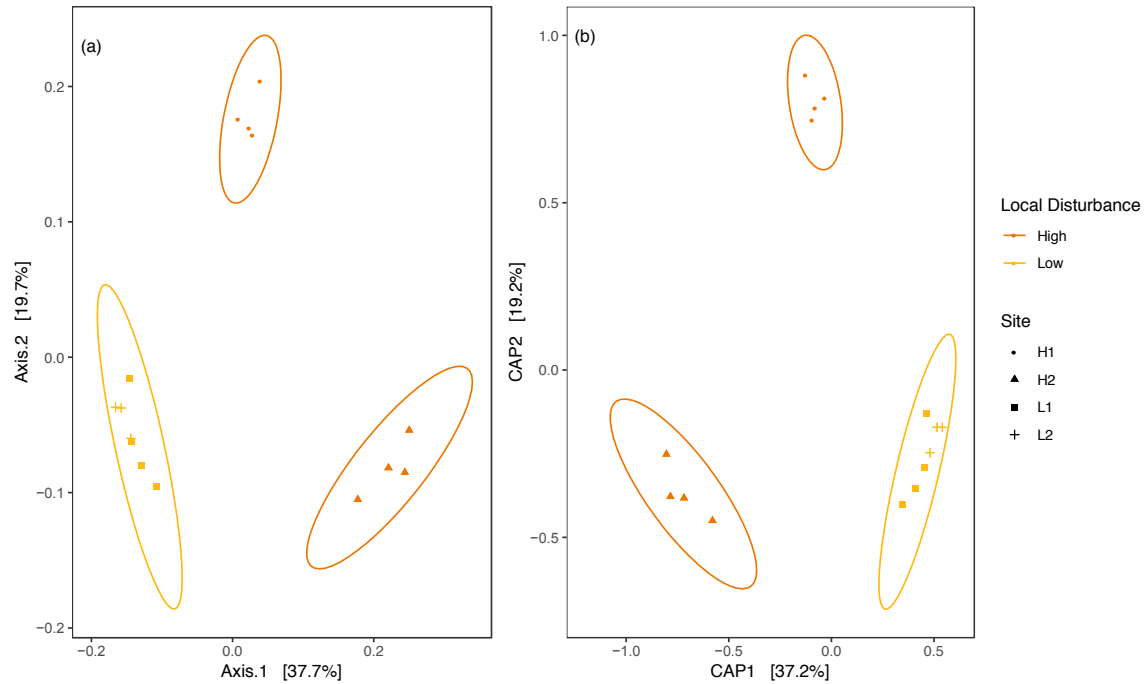


Figure S1.3. Differences in water microbial community composition amongst sites with different levels of local disturbance during high heat stress, demonstrated by (a) principal coordinates analysis of reef water samples at the high (orange) and low (yellow) disturbance levels for the high heat stress using Bray-Curtis distance. (b) Distance-based RDA of water samples at the high and low disturbance level, for the bleaching hotspot using Bray-Curtis distance (PERMANOVA, $F=13.94$, $p\text{-value}=0.0001$, $df=1$) (db-RDA, 77.3% of the variation explained). The ellipses are 95% confidence groupings.

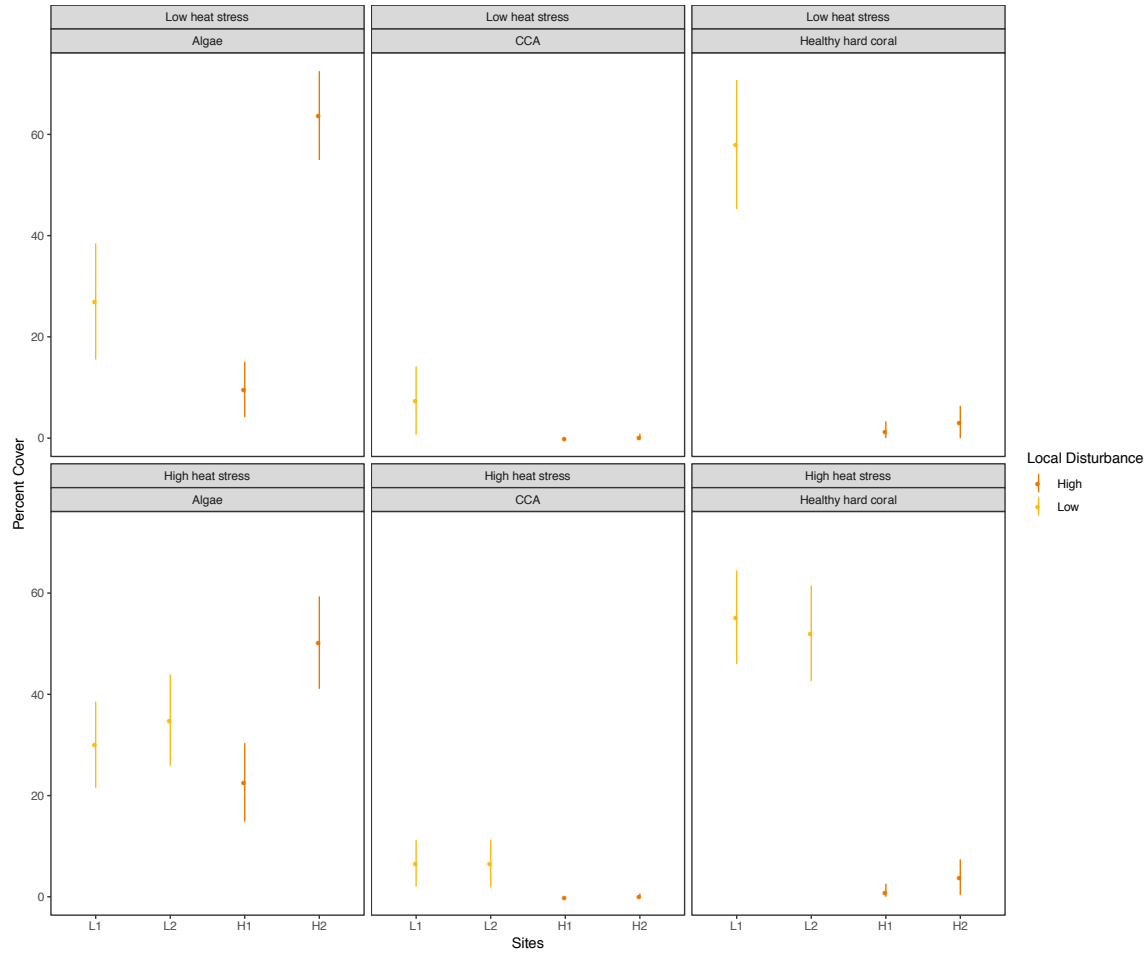


Figure S1.4. Benthic percent cover during the low and high heat stress for algae, crustose coralline algae, and healthy hard coral in the low (yellow) and high (orange) disturbance level. Error bars represent standard error. Percent cover is plotted by site to demonstrate any site variation.

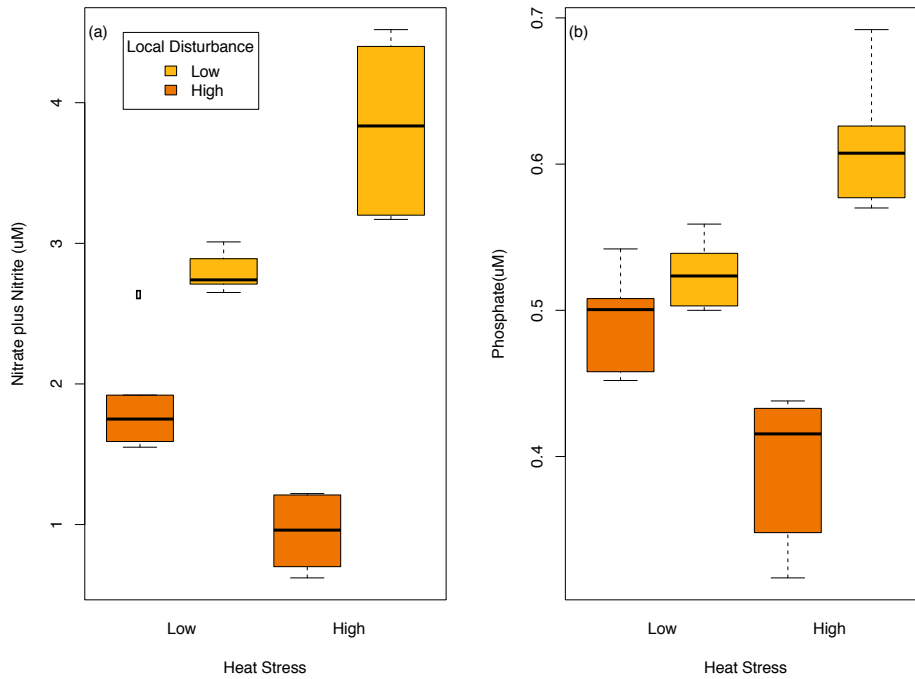


Figure S1.5. Nutrients at Kiritimati for each disturbance level (low=yellow, high=orange) and during each heat stress event (low and high) for (a) nitrate plus nitrite (uM) (Low Heat Stress: low=2.78 +/- 0.14 (SD), high=1.87 +/- 0.41; High Heat Stress: low=3.83 +/- 0.67, high=0.95 +/- 0.30) and (b) phosphate (uM) (Low Heat Stress: low=0.52 +/- 0.02, high=0.49 +/- 0.03; High Heat Stress: low=0.61 +/- 0.04, high=0.39 +/- 0.05).

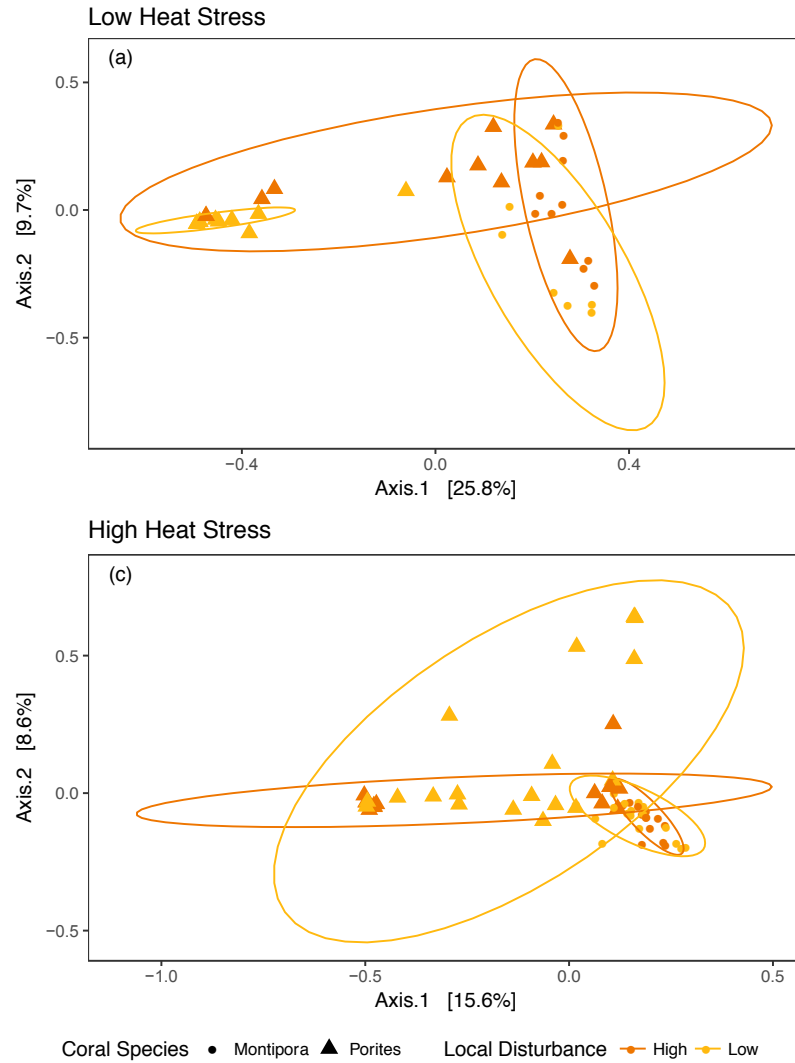


Figure S1.6. Differences in the microbial community of two coral species (*Montipora aequituberculata* (circle), *Porites lobata* (triangles): (a) at sites with high (orange) and low (yellow) local disturbance under low heat stress, as illustrated by a principal coordinates analysis (PCoA) using Bray-Curtis distance and (b) low and high local disturbance under high heat stress, as illustrated by a PCoA using Bray-Curtis distance. The ellipses are 95% confidence grouping

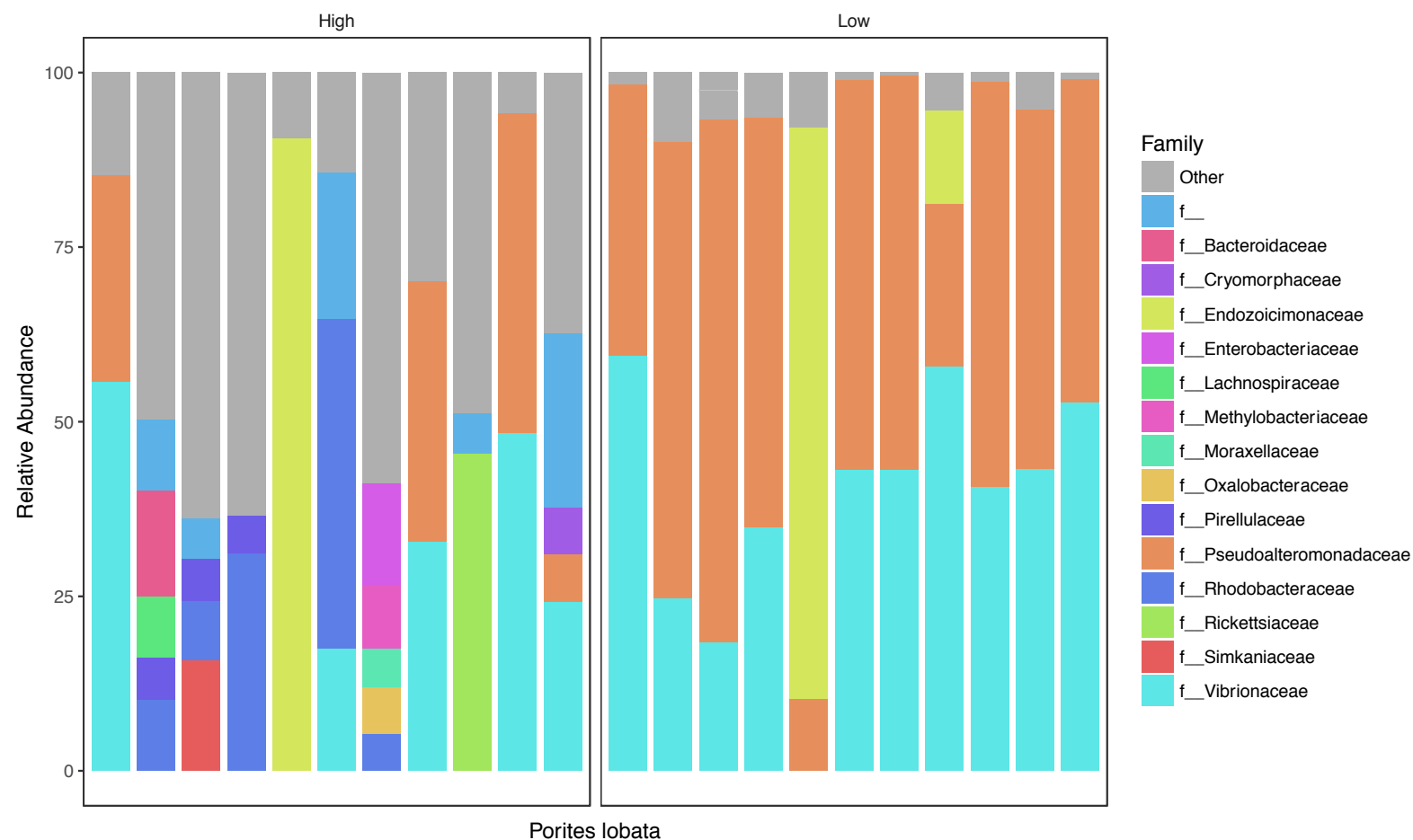


Figure S1.7. Relative abundance of microbial families for each *Porites lobata* sample during the low heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

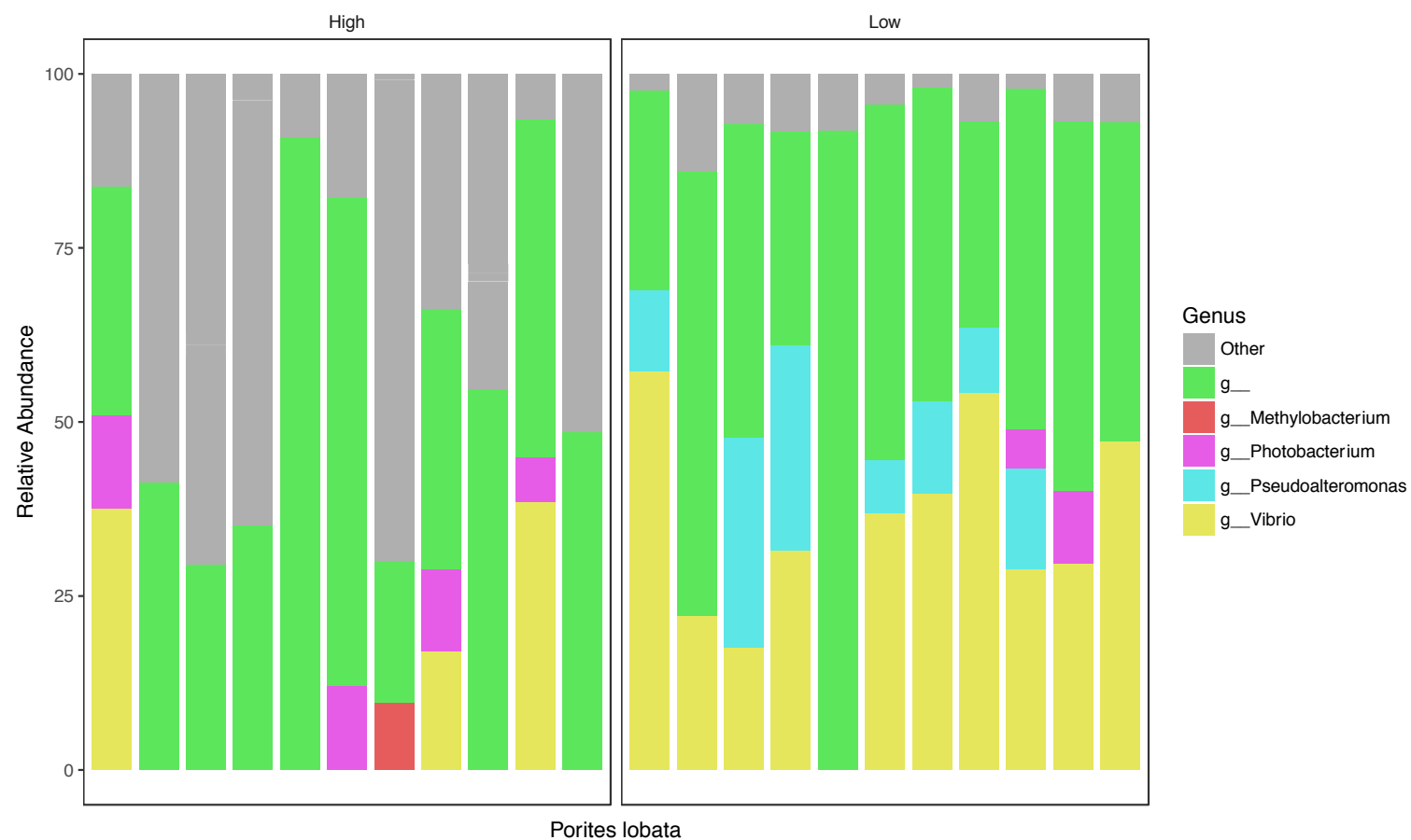


Figure S1.8. Relative abundance of microbial genera for each *Porites lobata* sample during low heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

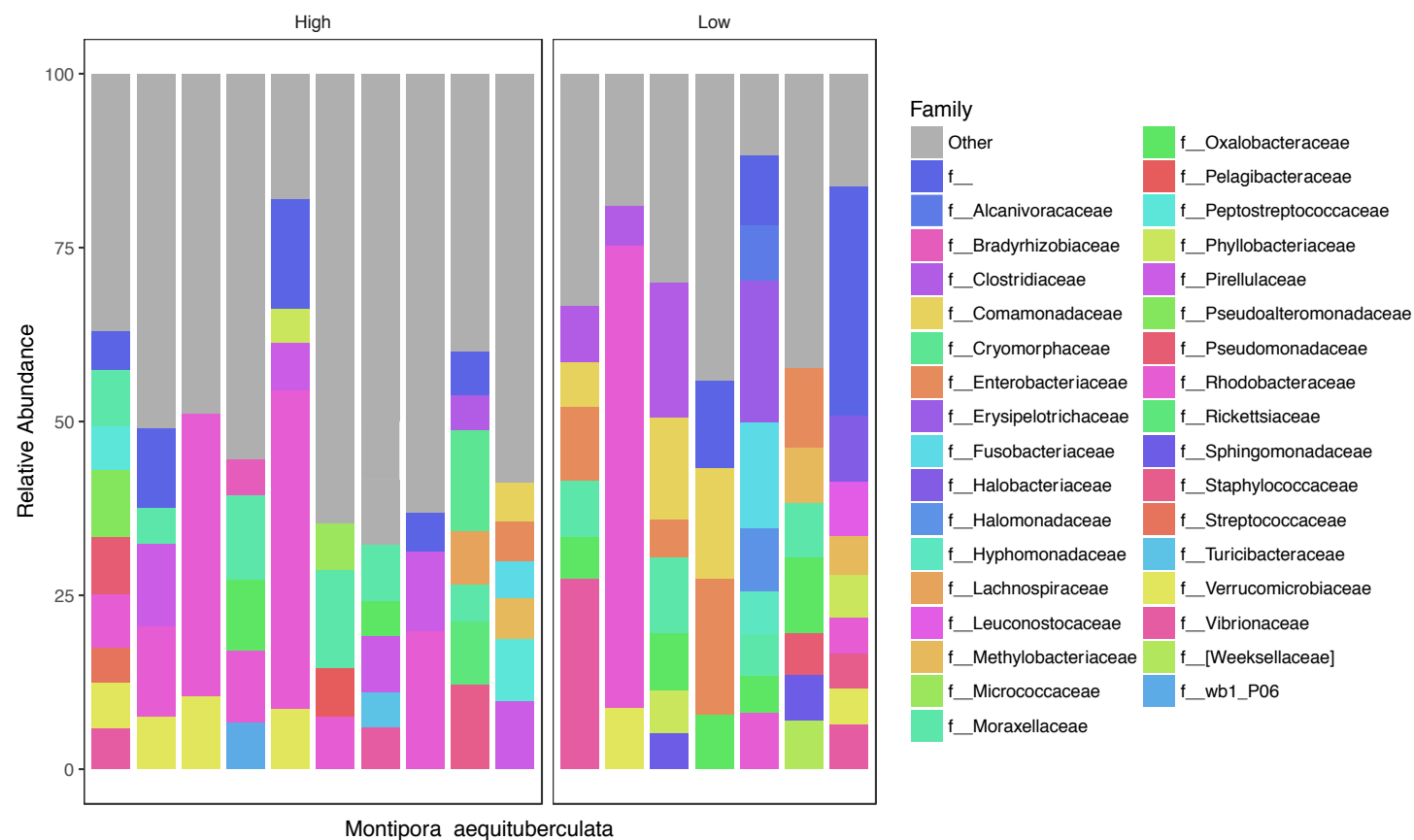


Figure S1.9. Relative abundance of microbial families for each *Montipora aequituberculata* sample during low heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

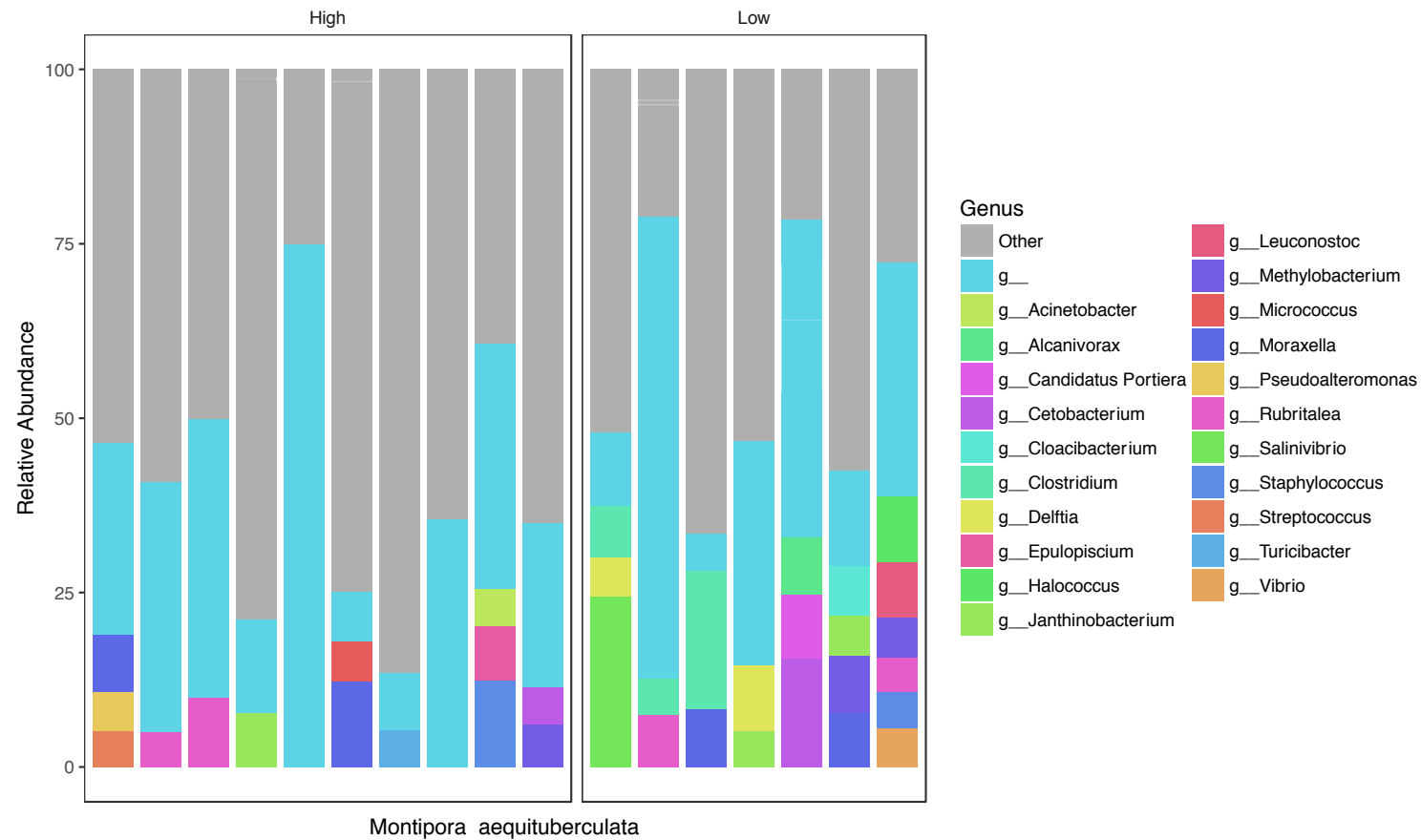


Figure S1.10. Relative abundance of microbial genera for each *Montipora aequituberculata* sample during low heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

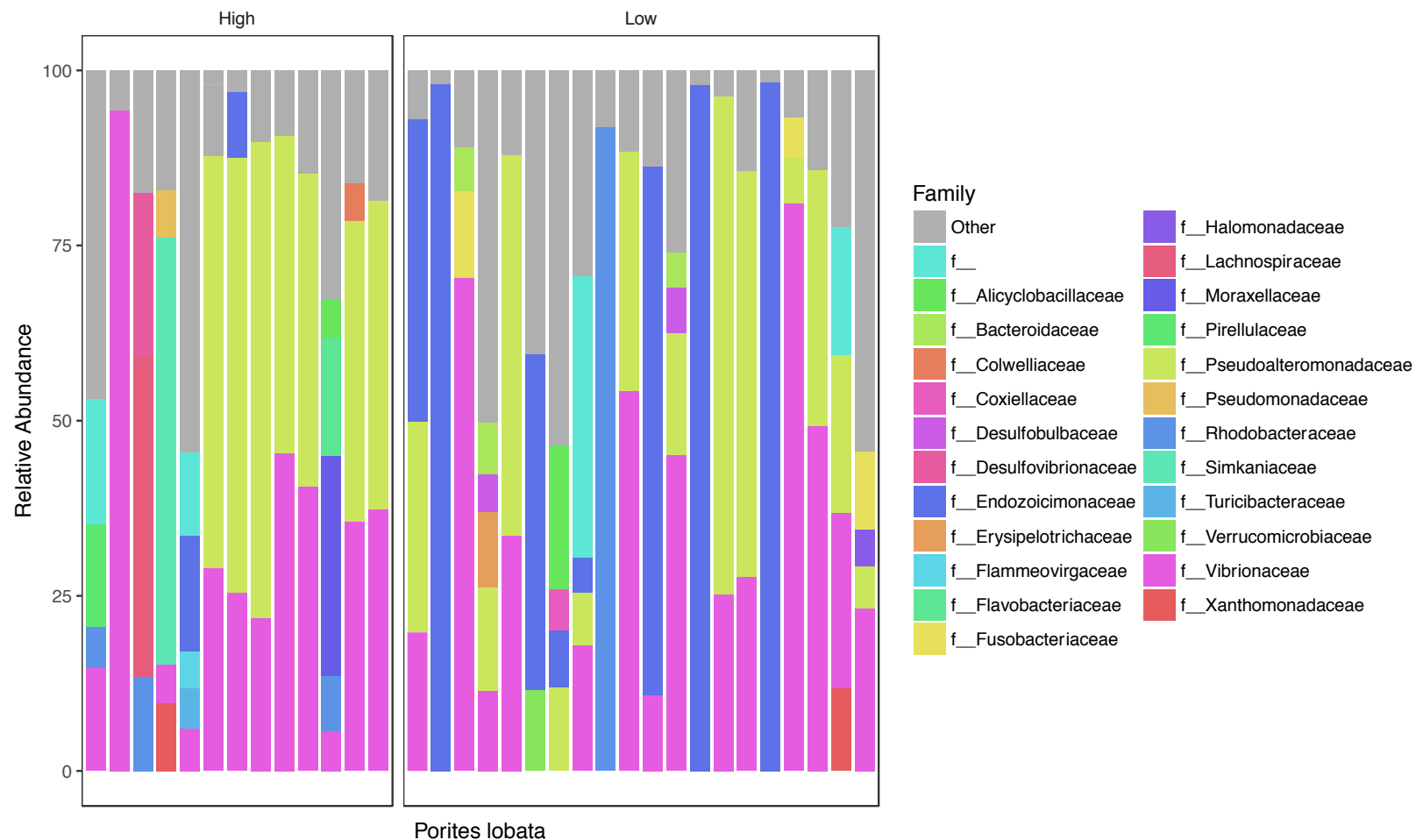


Figure S1.11. Relative abundance of microbial families for each *Porites lobata* sample during high heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

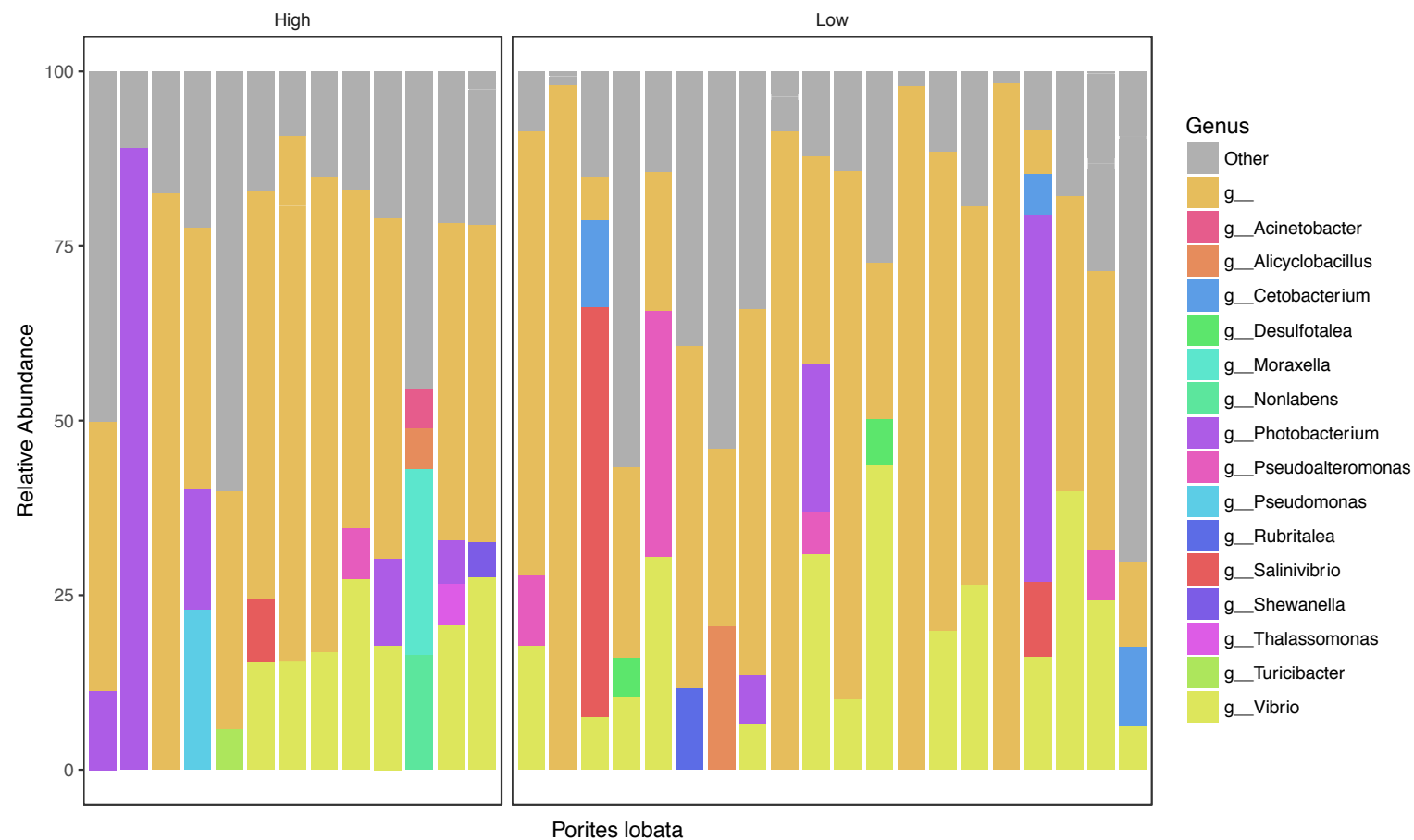


Figure S1.12. Relative abundance of microbial genera for each *Porites lobata* sample during high heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

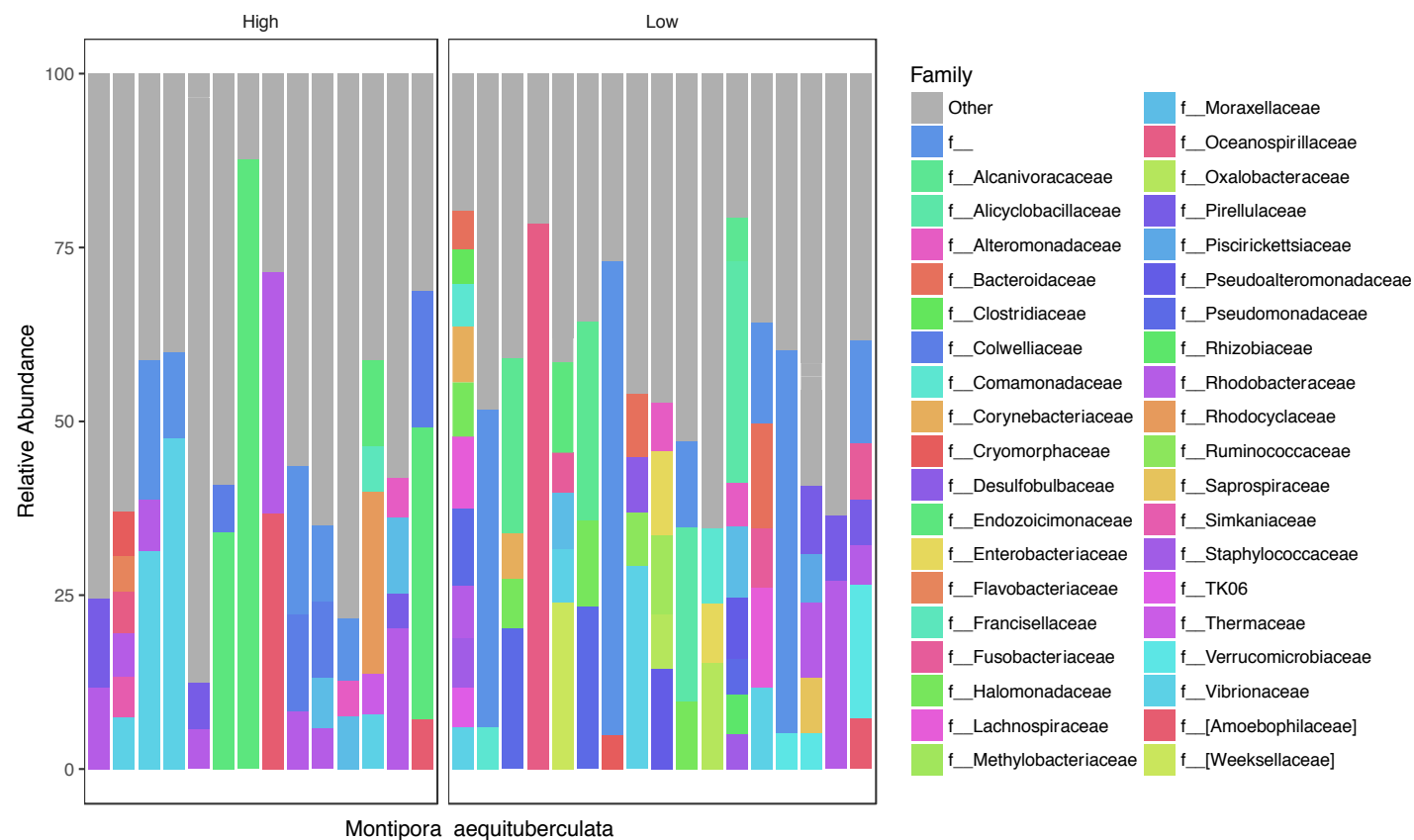


Figure S1.13. Relative abundance of microbial families for each *Montipora aequituberculata* sample during high heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any families in less than 5% relative abundance.

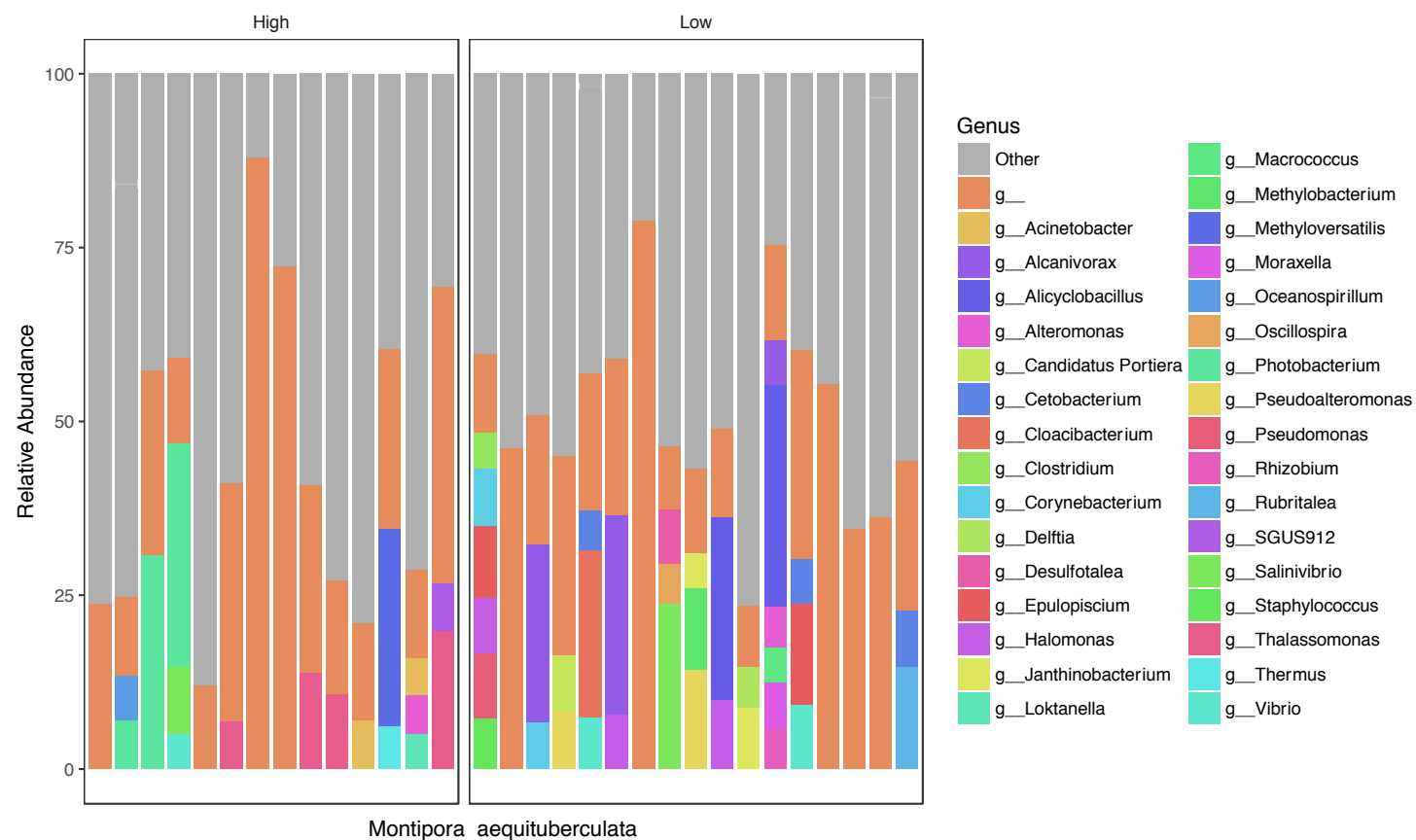


Figure S1.14. Relative abundance of microbial genera for each *Montipora aequituberculata* sample during high heat stress in high and low local disturbance level. Each bar is a coral sample. The grey colouring indicates any genera in less than 5% relative abundance.

Electronic Supplementary Material S2

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). We also conducted mock DNA extractions of no sample and just reagents.

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 μ m filtered and ultraviolet exposed water) were also included through these steps from the first PCR through to the equal molar pooling. The mock DNA extractions were checked after the first PCR and we saw no band or amplification in the gel. 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.

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_libraries_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 which did not match the reference database were then clustered together using de novo OTU picking (i.e. all at 97%). (Bokulich *et al.* 2013) suggest filtering spurious OTUS (i.e. errors) by filtering OTUs not found at 0.005%. However, this suggestion was for forward reads, therefore we increased the phred quality score from the suggestion of 3 to 20 and have paired-end reads (i.e. thus increasing the quality), and 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. Sequences identified as mitochondria and chloroplasts were filtered out.

As coral samples are low biomass, and their mitochondria amplify with 16S primers, the samples were compared to the six negative controls that were sequenced in the same run (Electronic Supplementary Material S2). Taxa identified within the

Montipora aequituberculata 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, you never capture all the diversity, so it is better to be conservative for our analysis.

Electronic Supplementary Material S3

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.

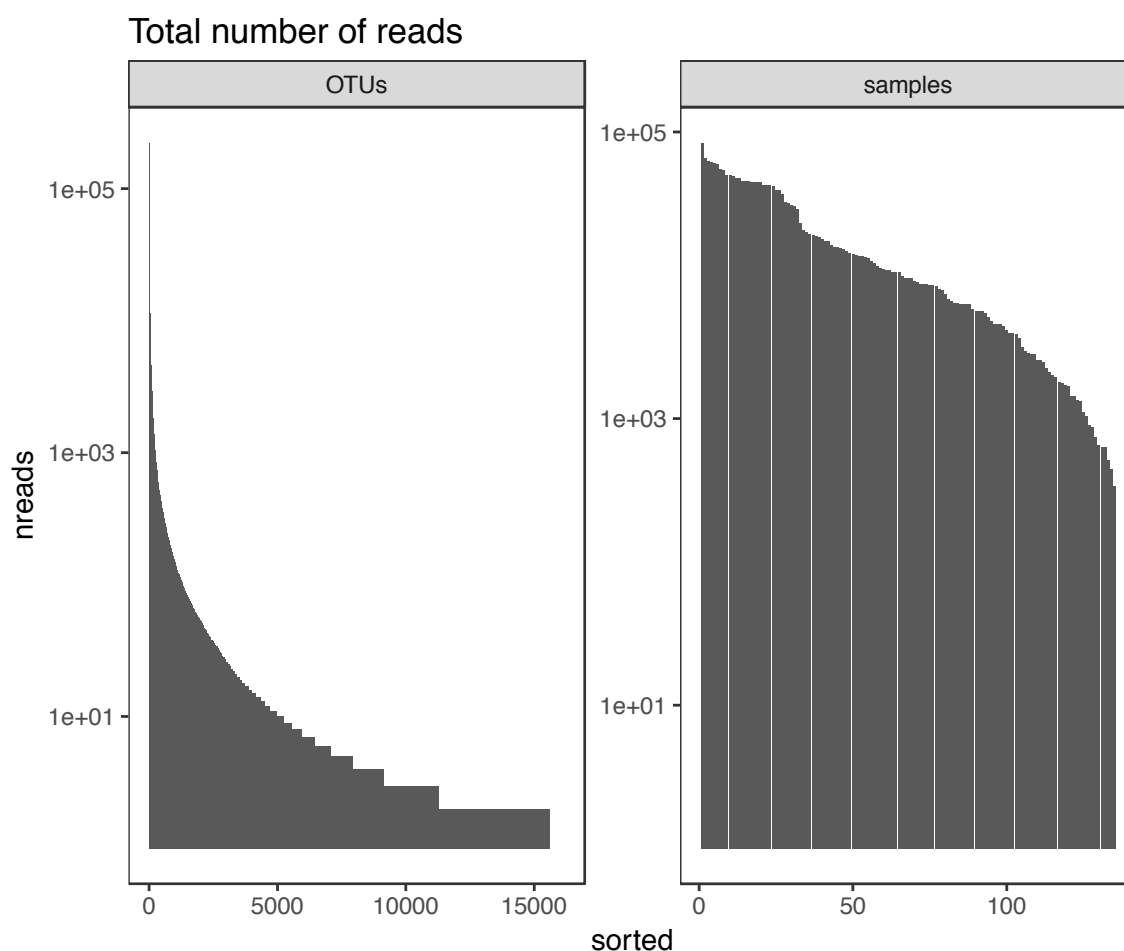


Figure S3.1. The total number of reads for each OTU and for each sample (water and coral) prior to contamination filtering but after pre-processing (i.e. filtering out mitochondria and chloroplasts).

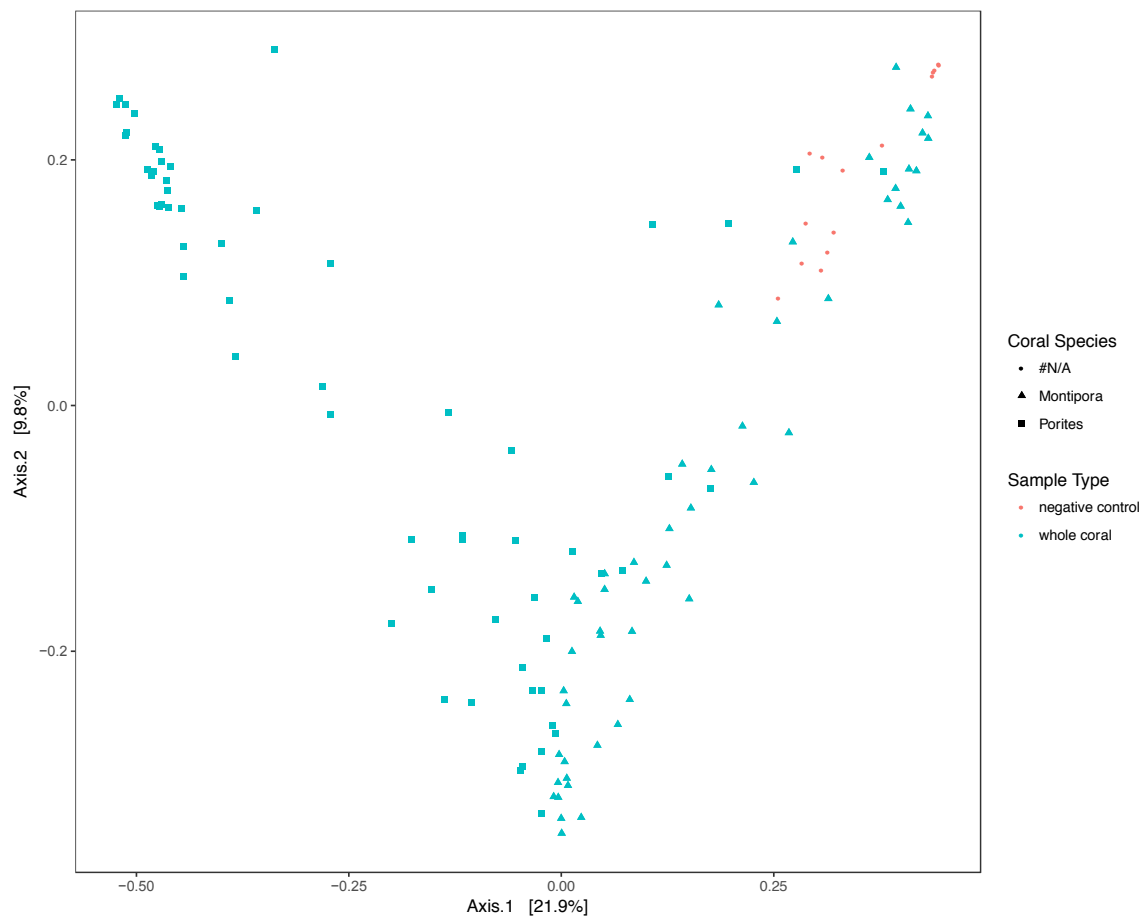


Figure S3.2. Principal coordinate analysis demonstrating the similarity of the negative controls (pink) with coral samples (blue) especially *Montipora aequituberculata* (triangle shape) using the Bray-Curtis dissimilarity index.

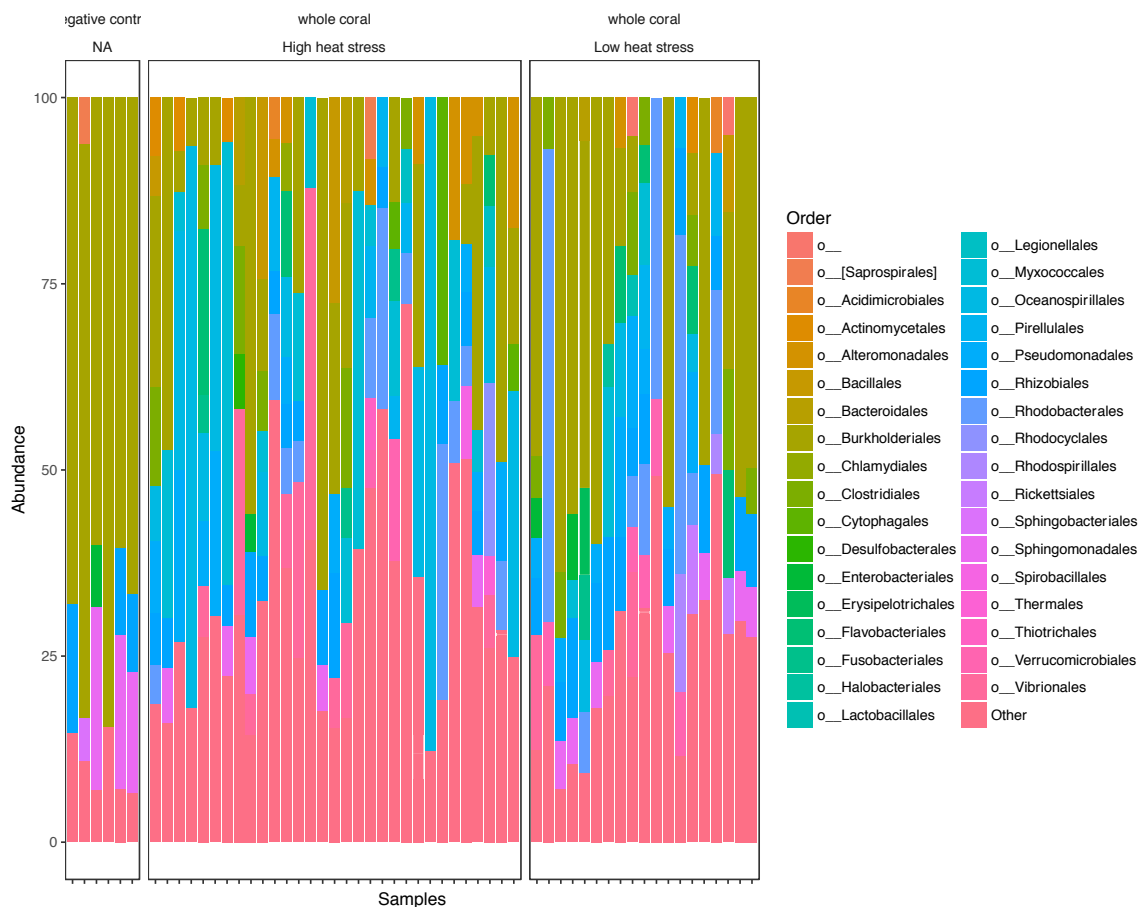


Figure S3.3. Bar plot of bacterial relative abundance for the six negative controls and *Montipora aequituberculata* (i.e. the coral with the highest amount of OTUs similar to contamination). Each bar is a sample. Any orders with less than 5% relative abundance are placed into the “Other” category.

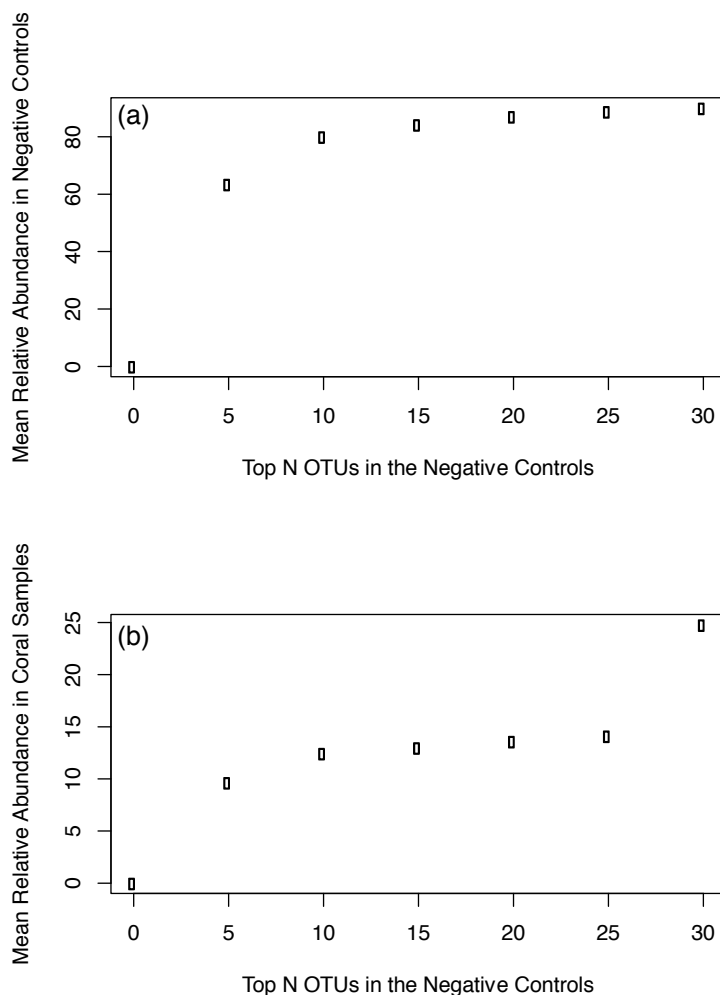


Figure S3.4. (a) The mean relative abundance in negative controls of the top 0-30 OTU's found in the negative controls. Note: there were 15 negative controls as there were technical replicates of the negative controls that were sequenced. To determine the average top N OTUs within the negative controls, 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").

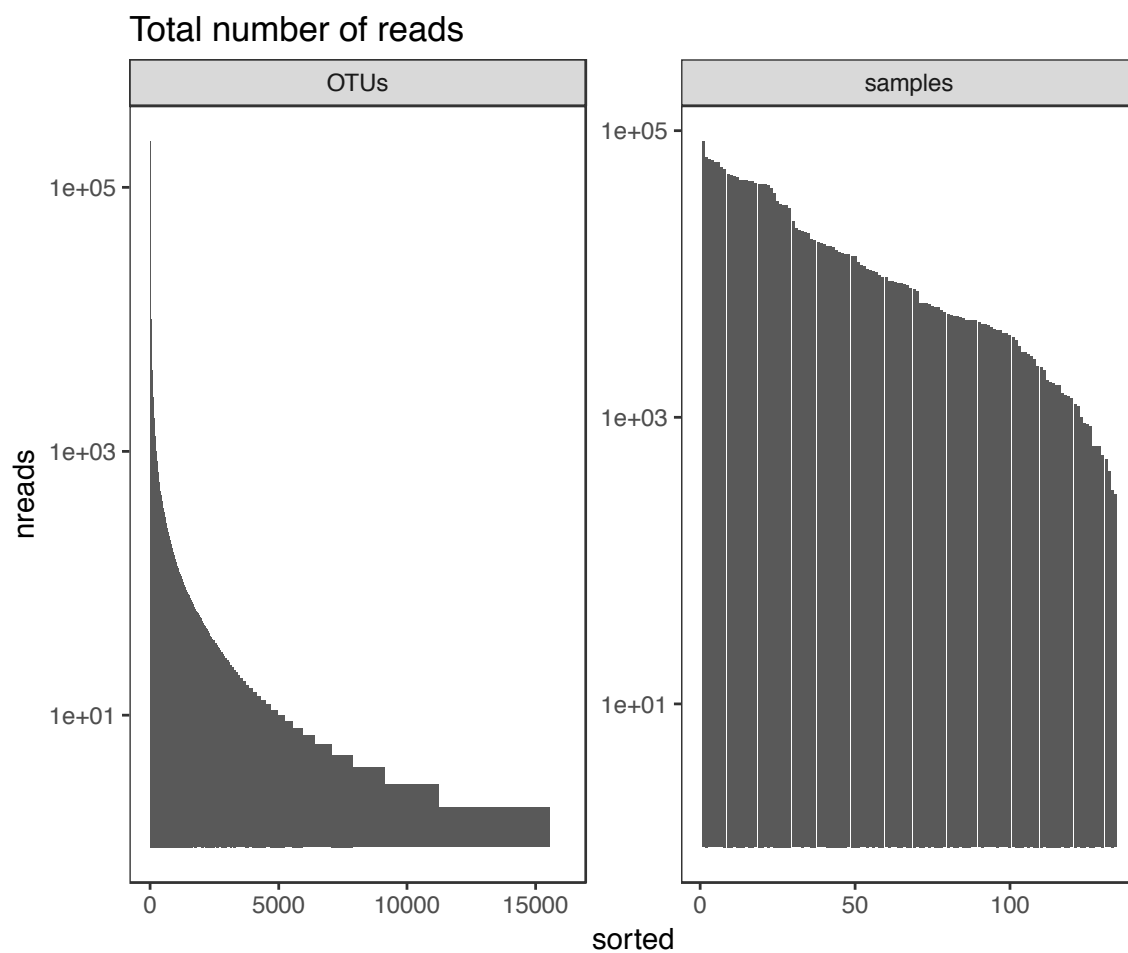


Figure S3.5. The total number of reads for each OTU and for each sample after filtering out the top 10 OTUs in the negative controls.

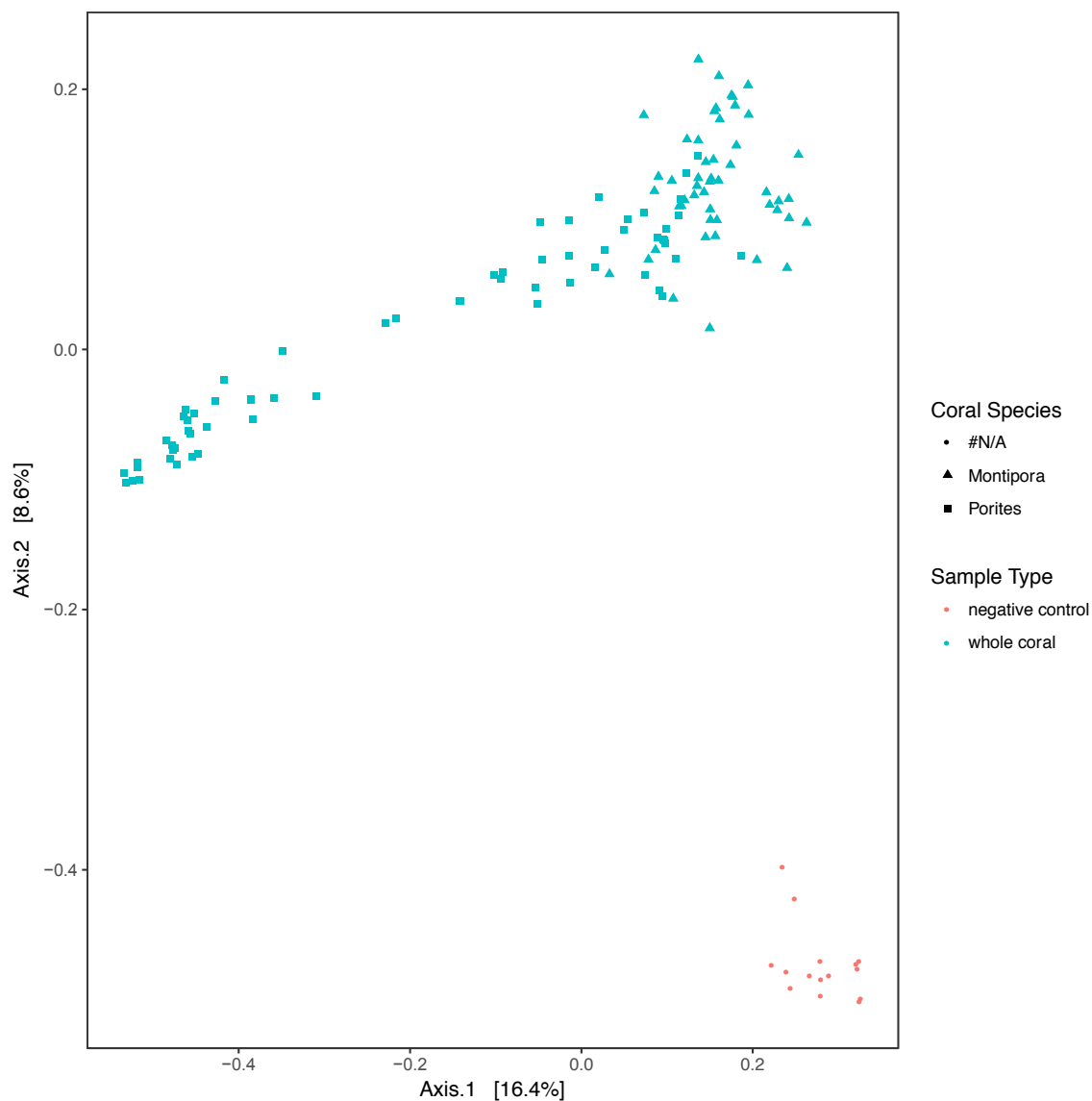


Figure S3.6. Principal coordinates analysis demonstrating how filtering the top 10 OTUs found within the negative controls separates the negative controls (pink) from the coral samples (blue) using the Bray-Curtis dissimilarity.

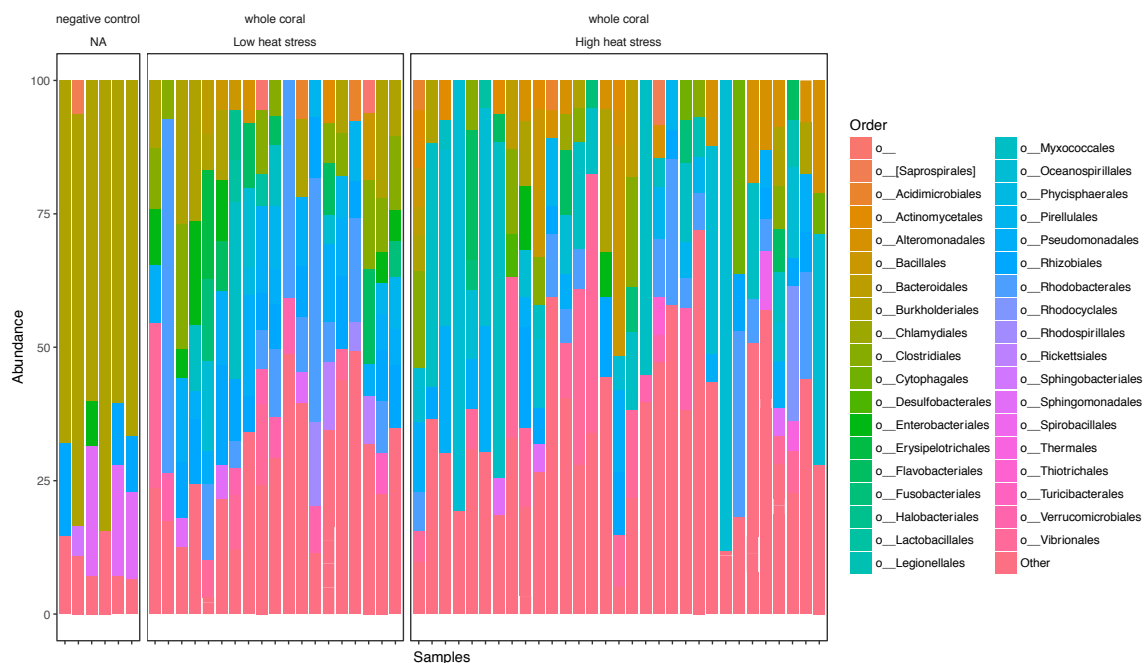


Figure S3.7. Bar plot of bacterial relative abundance for the old negative controls and the new *Montipora aequituberculata* (i.e. with the top 10 OTUs filtered). Each bar is a sample. Any orders with less than 5% relative abundance are placed into the “Other” category.

Table S3.1. 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__