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Title: Diversity and stability of coral endolithic microbial communities at a naturally high pCO$_2$ reef.

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Abstract:

The health and functioning of reef-building corals is dependent on a balanced association with prokaryotic and eukaryotic microbes. The coral skeleton harbours numerous endolithic microbes, but their diversity, ecological roles and responses to environmental stress, including ocean acidification, are not well characterized. This study tests whether pH affects the diversity and structure of prokaryotic and eukaryotic algal communities associated with skeletons of *Porites* spp. using targeted amplicon (16S rRNA gene, UPA and *tuf*A) sequencing. We found that the composition of endolithic communities in the massive coral *Porites* spp. inhabiting a naturally high pCO$_2$ reef (avg. pCO$_2$ 811μatm) is not significantly different from corals inhabiting reference sites (avg. pCO$_2$ 357μatm), suggesting that these microbiomes are less disturbed by ocean acidification than previously thought. Possible explanations may be that the endolithic microhabitat is highly homeostatic, or that the endolithic microorganisms are well adapted to a wide pH range. Some of the microbial taxa identified include nitrogen-fixing bacteria (*Rhizobiales* and cyanobacteria), algicidal bacteria in the phylum *Bacteroidetes*, symbiotic bacteria in the family *Endozoicomoniaceae*, and endolithic green algae, considered the major microbial agent of reef bioerosion. Additionally, we test whether host species has an effect on the endolithic community structure. We show that the endolithic community of massive *Porites* spp. is substantially different and more diverse than that found in skeletons of the branching species *Seriatopora hystrix* and *Pocillopora damicornis*. This study reveals highly diverse and structured microbial communities in *Porites* spp. skeletons that are possibly resilient to ocean acidification.

Introduction

Ocean acidification (OA) is predicted to threaten the persistence of coral reefs by affecting the balance between constructive forces (calcification and growth of reef builders) and destructive forces (bioerosion and carbonate dissolution) (Tribollet 2008a; Andersson & Gledhill 2013). Acidification lowers the saturation state of calcium carbonate ($CaCO_3$), making it more difficult for calcifying organisms, such as stony corals, to build their skeletons (Orr *et al.* 2005; Hofmann *et al.* 2010). OA has been shown to slow down calcification and cause structural deformities in juvenile corals (Crook *et al.* 2013; Foster *et al.* 2016). Some studies, however, indicate that corals are able to regulate the pH at the tissue-skeleton interface, where calcification takes place, mitigating the potential consequences of OA on the calcification process (McCulloch *et al.* 2012; Venn *et al.* 2013; Georgiou *et al.* 2015). Rates of biological dissolution of $CaCO_3$ (bioerosion) tend to increase under low pH conditions, mostly due to an increase in biomass of the boring organisms living inside coral skeletons (Manzello *et al.* 2008; Tribollet *et al.* 2009; Crook *et al.* 2013; Fang *et al.* 2013; Reyes-Nivia *et al.* 2013; Enochs *et al.* 2016), potentially resulting in a shift from a net reef accretion condition to one of net erosion (Andersson & Gledhill 2013).

The skeletons of live and dead corals harbour bacteria, fungi, sponges and an abundant population of limestone-boring algae, all having important roles in the reef's $CaCO_3$ budget (Le Campion-Alsumard *et al.* 1995; Tribollet 2008a; Verbruggen & Tribollet 2011). For example, the green alga *Ostreobium* can be responsible for 70–90% of carbonate dissolution within dead corals, eroding as much as 1 kg of reef carbonate per m^2 per year (Tribollet 2008b; Grange *et al.* 2015). Green algal biomass in live coral skeletons exceeds *Symbiodinium* biomass in coral tissues by about 16 times (Odum & Odum 1955), making the limestone attractive to grazers and further increasing bioerosion (Chazottes *et al.* 1995; Clements *et al.* 2016). However, endolithic algae also protect corals from high light stress (Yamazaki *et al.* 2008) and provide vital nutrients to corals, potentially extending the time they can survive without *Symbiodinium* during bleaching events (Schlichter *et al.* 1995; Fine & Loya 2002). Endolithic algae have exceptionally high levels of cryptic diversity (Marcelino & Verbruggen 2016; Sauvage *et al.* 2016; Del Campo *et al.* 2017), and although it is known that their biomass increases substantially upon acidification and warming (Tribollet *et al.* 2009; Reyes-Nivia *et al.* 2013), it is not known which of the cryptic species increase in relative abundance.

The endolithic community, along with the coral host and its other symbionts, constitutes the coral holobiont (Rohwer *et al.* 2002). The responses of the coral microbiome, including both prokaryotic and eukaryotic members, to acidification has gained attention as we continue to uncover vital roles played by microorganisms in holobiont health and resilience (Bourne *et al.* 2009; Sharp & Ritchie 2012; Krediet *et al.* 2013; Blackall *et al.* 2015; Bourne *et al.* 2016). Because the ocean pH naturally changes throughout seasons, along depth gradients, with productivity and other biological factors, marine microorganisms may have the physiological plasticity required to cope with the predicted levels of ocean acidification over the next 100 years (Joint *et al.* 2011). This notion is supported by several studies showing stable coral prokaryotic community when shifted from ambient to high CO_2 partial pressure (pCO_2) and therefore reduced seawater pH conditions (Meron *et al.* 2012; Webster *et al.* 2016; Zhou *et al.* 2016). However other studies have demonstrated that a reduced seawater pH can lead to the loss of *Symbiodinium* (coral bleaching) and trigger shifts from a healthy microbiome composition to a microbial community typically associated with diseased corals (Anthony *et al.* 2008; Vega Thurber *et al.* 2009; Meron *et al.* 2011; Webster *et al.* 2013; Morrow *et al.* 2015). These different responses of a coral's microbiome to reduced seawater pH may reflect differences in resilience across coral species to acidification or different experimental setups used in the various studies.

Reefs at the Milne Bay Provence of Papua New Guinea (PNG) are in close proximity to volcanic seeps (expelling ~ 99% pure CO_2) and constitute a good model system to study the impacts of acidification *in situ* on the microbial community associated with corals. Both coral species composition and the prokaryotic microbial community associated with coral tissue and mucus differ between high pCO_2 seep sites and nearby reference sites with ambient pCO_2 (Fabricius *et al.* 2011; Morrow *et al.* 2015). However, little is known about the coral endolithic communities and how these may change under various seawater pH conditions. Previous studies have screened 16S rRNA gene clone libraries and demonstrated contrasting results, with significant effects of OA community composition within the skeleton in an experimental system (Meron *et al.* 2011) but no significant changes in corals transplanted to a natural CO_2 seep site (Meron *et al.* 2012). One limitation with the 16S rRNA gene marker is that it underestimates the diversity of eukaryotic algae (Marcelino & Verbruggen 2016), and as a consequence, the major microbial agents of bioerosion have been overlooked.

Here we use high-throughput amplicon sequencing to investigate the effects of ocean acidification on the diversity and structure of endolithic microbial communities of corals inhabiting a high pCO_2 site in PNG. Our goals are to: 1) test whether the community composition of prokaryotes and photosynthetic eukaryotes (assessed with the 16S rRNA gene, UPA and *tuf*A markers) within the skeletons of massive colonies of *Porites* spp. differs between high pCO_2 sites and nearby reference sites where pCO_2 is not affected by the volcanic seeps; 2) compare the endolithic communities of *Porites* spp. with those of two branching coral species (*Seriatopora hystrix* and *Pocillopora damicornis*) to investigate whether the microbiome in coral skeletons varies among host species; and 3) describe the endolithic community diversity found in corals of Papua New Guinea and discuss the potential functional roles of this microbiome under ocean acidification.

Methods

Field sites and sampling

Samples of massive *Porites* spp. (n = 24, six per site and month) were collected in April and November 2014 at two high pCO_2 (seep) and reference sites within the D'Entrecasteaux Islands, Milne Bay Province, Papua New Guinea. High pCO_2 samples were collected at Illi Illi (Upa-U) Seep (09.82425S, 150.81789E) and Dobu Seep (09.73646S, 150.86894E), and at nearby reference sites (ambient pCO_2) not exposed to elevated pCO_2 conditions (Illi Illi Reference, 09.82806S, 150.82028E and Dobu Reference, 09.75211S, 150.85410E) (Fabricius *et al.* 2011; Uthicke *et al.* 2013). High pCO_2 and reference sites were ~500 m and ~3 km apart from one another at Illi Illi and Dobu respectively. Samples of the branching corals *Seriatopora hystrix* (n = 3 at each site) and *Pocillopora damicornis* (n = 3 at each site) were only collected in April 2014 at Illi Illi seep and reference sites (same as above). Seawater carbonate chemistry varies in response to bubble activity and water motion at the seep sites; thus, at Illi Illi seep, corals experience a pH range (defined here as the 5th and 95th percentiles) of 7.28–8.01 (avg. pCO_2 624 μatm) and at Dobu seep a pH range of 7.08-7.99 (avg. pCO_2 998 μatm). At Illi Illi reference site the pH ranges from 7.91–8.09 (avg. pCO_2 346 μatm) and at Dobu reference site the pH ranges from 7.91–8.10 (avg. pCO_2 368 μatm) (Fabricius *et al.* 2014), which is within the range of future predictions for the year 2100 (Moss *et al.* 2010).

Coral fragments were collected using bone cutters or a hammer and chisel and placed into individual sections within a plastic tackle box, which allowed for water flow whilst underwater. After returning to the boat, samples were immediately placed into flowing seawater sourced directly from the collection site. Large pieces of *Porites* spp. were chipped into smaller fragments, rinsed thoroughly with sterile 0.02 µm-filtered seawater and placed in 50 mL Falcon tubes with RNAlater (Ambion). Samples were kept in a cooler with ice until returned to the laboratories at the Australian Institute of Marine Science (AIMS) where they were processed.

Fragments were removed from RNAlater and soaked in 0.2 µm filtered calcium and magnesium free seawater for ~10 minutes at room temperature (CMFSW; 0.45M NaCl, 10mM KCl, 7mM Na_2SO_4, 0.5mM $NaHCO_3$ and milli-Q water; (Esteves *et al.* 2016)) to aid in the initial removal of tissues from the skeleton. Tissues were removed into the CMFSW using an air gun fitted with a sterile tip. Skeletons with tissues removed were placed back into the original RNAlater collection buffer and stored at -80°C until shipment to the University of Melbourne where DNA was isolated from the endolithic community (see below).

DNA isolation, library preparation and sequencing

Total DNA was isolated from coral skeletons using the Wizard Genomic DNA Purification Kit (Promega). The manufacturer's protocol for plant DNA was followed, with the exception of an extended 3 hr incubation step with the first extraction buffer to allow the DNA to leak out from the limestone into the solution. Amplified DNA products for library preparation were obtained with a two-step process described by Marcelino and Verbruggen (2016). During the first PCR step, three metabarcoding markers were amplified: the 16S rRNA gene (Klindworth *et al.* 2013); the universal plastid amplicon (UPA), which is a fragment of the 23S rRNA gene (Presting 2006; Sherwood & Presting 2007) and the elongation factor Tu (*tuf*A), which targets green algae (Ulvophyceae) (Fama *et al.* 2002; Marcelino & Verbruggen 2016). During the second PCR step, barcodes and Illumina adapters were attached to both 3' and 5' ends of the amplicons. One negative control was performed with each amplification (6 in total, one per marker and per amplification step) and sequenced with the library, even though no DNA was detected in any negative control during quantification. Two mock 'blank' extractions were also performed along with the samples DNA isolation, processed through the amplification process and sequenced to further control for possible contamination. DNA isolation and PCR preparation were carried out inside a dedicated dead-air box (PCR workstation) sterilized with UV light for 15 min prior to each use. Libraries were quantified using the Quant-It PicoGreen reagent (Invitrogen) and pooled with other samples of another project. The library was sequenced using the Illumina MiSeq platform (2×300 bp paired end reads) at the Centre for Translational Pathology, University of Melbourne. Further details about the primers and library preparation are provided in Supplementary materials.

Data processing

The MiSeq run yielded one file containing all amplicons per sample, which were demultiplexed based on the primer sequences. The 3' ends of reads were trimmed to improve consensus quality; forward and reverse reads were merged using FLASH (Magoc & Salzberg 2011) and sequences having average quality scores smaller than 35 or lengths shorter than a threshold (350 bp for 16S rRNA gene, 320 bp for UPA and 400 bp for the *tuf*A) were filtered out using PRINSEQ (Schmieder & Edwards 2011). To verify that the data lost during quality control did not affect the results, we also performed the analyses with a less stringent quality filtering (Supplementary materials). Sequences were clustered into Operational Taxonomic Units (OTUs) using UPARSE (Edgar 2013). A similarity threshold of 98% was set for the *tuf*A marker, a threshold near species level for this marker (Sauvage *et al.* 2016). For the other markers, the default threshold of 97% was used. The 16S rRNA gene OTUs were aligned with PyNAST (Caporaso *et al.* 2010a) while the UPA and *tuf*A datasets were aligned with MAFFT (Katoh *et al.* 2002). A taxonomy affinity was assigned to the OTUs using the Naïve Bayesian Classifier (RDP) implemented in QIIME v.1.9.1 (Wang *et al.* 2007; Caporaso *et al.* 2010b). The Greengenes v.13.8 dataset (DeSantis *et al.* 2006) was used to classify the 16S rRNA gene sequences, and custom-made reference datasets (described and available in Marcelino & Verbruggen 2017) were used for *tuf*A and UPA. The resulting OTU table went through a filtering process to remove OTUs found in the negative controls (3 OTUs in the 16S rRNA gene, 5 OTUs in the UPA and 2 OTUs in the *tuf*A datasets) and rare OTUs (i.e. OTUs with less than 5 reads across all samples and OTUs from samples where they are present with 2 or less reads). OTUs were also filtered based on their taxonomic classification to focus on the taxonomic groups that each marker best characterizes: chloroplast sequences were excluded from the 16S rRNA gene dataset and bacterial sequences were excluded from the *tuf*A dataset. Further details about the data processing pipeline are provided in the Supplementary materials.

Statistical analysis

There were no significant differences related to the time of collection (Supplementary materials), therefore all *Porites* spp. samples (n = 24) were used to investigate the effects of $p\mathrm{CO}_2$ in endolithic communities associated with this coral genus. Rarefaction curves of the number of observed OTUs per number of reads were constructed by randomly subsampling the reads in QIIME, allowing to set a threshold for each marker where the curve reaches saturation (i.e. a plateau in the rarefaction curve), which was 2,200 reads in the 16S rRNA gene, 1,400 in the *tuf*A and 7,000 in the UPA dataset (Supplementary figure 1). Samples with sequencing depths lower than these thresholds were excluded, resulting in 20 samples in the 16S rRNA gene, and 22 samples in the UPA and *tuf*A datasets (Supplementary Table S1). Alpha diversity indices (Chao1 and observed OTUs) were calculated using QIIME (Caporaso *et al.* 2010b). The relative abundance of individual OTUs and taxonomic groups (i.e. OTUs grouped at phylum level for bacteria and genus level for algae) between sites were tested for significant differences with a Kruskal–Wallis test (for OTUs) and ANOVA (for taxon groups) using QIIME (Caporaso *et al.* 2010b).

To further investigate the distribution of green algal lineages, a maximum likelihood phylogeny was built with the green algal *tuf*A OTUs together with reference sequences (from GenBank) using a GTR+gamma model of sequence evolution in RAxML v.8.2.6 (Stamatakis 2006).

OTUs present in less than 3 samples were excluded, their relative abundances were normalized with cumulative sum scaling (Paulson *et al.* 2013) and visualized alongside a phylogenetic tree using the R package phytools (Revell 2012).

Potential differences in community composition between high $p\mathrm{CO}_2$ and reference sites (beta-diversity) were investigated with a combination of statistical methods. Principal coordinate analysis (PCoA) on weighted UniFrac distance matrices was performed using QIIME (Caporaso *et al.* 2010b; Lozupone *et al.* 2011) and the results visualized using the ggplot2 package in R (Wickham 2009). A multivariate generalized linear model (MGLM) was used to investigate potential differences in community composition between high $p\mathrm{CO}_2$ and reference sites. The MGLM was computed using the mvabund R package (Wang *et al.* 2012), considering a negative binomial distribution. The null hypothesis of no difference among sites was statistically tested with analysis of deviance using 999 bootstrap interactions (R scripts provided in Supplementary materials). To verify that the results are not a consequence of PCR bias, PCoA and MGLM analyses were also performed with a distance matrix based on Sørensen similarity, which is a presence/absence index (Supplementary materials).

The number of samples of the branching species (*S. hystrix* and *P. damicornis*) did not allow statistical analyses to test for differences between high $p\mathrm{CO}_2$ and reference sites, although it did permit a comparison of the endolithic communities associated with the different coral hosts (Supplementary Table 1). To investigate the community structure related to host species, a rarefaction threshold of 707 reads for the 16S rRNA gene, 713 for the *tuf*A and 3257 for the UPA marker was used, allowing the inclusion of a larger number of samples in the analysis. Samples with sequencing depths lower than these thresholds were excluded, resulting in 35 samples in the 16S rRNA gene and UPA datasets and 27 in the *tuf*A datasets (Supplementary Table S1). Alpha diversity, Kruskal–Wallis test (for OTUs), ANOVA (for taxon groups), PCoA and MGLM were performed on this dataset as previously described, but here, samples from different $p\mathrm{CO}_2$ conditions from conspecific host species were combined in order to investigate the community structure purely associated with coral host species.

Results

Effects of $p\mathrm{CO}_2$ conditions on the Porites spp. endolithic microbiome

A total of 6,584,274 sequence reads were recovered for the samples analysed here, 4,405,336 belonging to *Porites* spp. samples (*ca.* 25% of these reads belonged to a marker that is not analysed in this manuscript). After stringent pre and post OTU-clustering and filtering, a total of 119,367 (16S rRNA gene), 109,948 (*tuf*A) and 393,816 (UPA) reads remained in the analysed *Porites* spp. dataset. A less stringent quality filtering which resulted in inclusion of more sequence reads did not alter the community patterns (see Supplementary materials). The alpha diversity statistics, including Chao1 and observed OTUs, indicated that the species richness of the endolithic communities associated with *Porites* spp. was not significantly different between high $p\mathrm{CO}_2$ and reference sites (Table 1). Although the relative abundance of some microbial taxa differed between high $p\mathrm{CO}_2$ and reference sites (Figure 1, see also Supplementary figure 2 for a sample-based representation), the differences were not statistically significant (Bonferroni-corrected *p*-values = 1), neither at the OTU level (Supplementary table S2) nor at higher taxonomic levels (Supplementary table S3). Accordingly, principal coordinate analysis and MGLM did not reveal any significant pattern between sites with all three markers (Figure 2).

We further investigated whether any of the different phylogenetic lineages in the endolithic algal communities differed in abundance at high $p\mathrm{CO}_2$ and reference sites. A phylogenetic heatmap of relative abundances (Supplementary figure 3) indicated that phylogenetic relatedness among green algae is not correlated with different abundances in high $p\mathrm{CO}_2$ or reference sites. Notably, three algal OTUs were present in either high $p\mathrm{CO}_2$ or reference sites, but not in both (Supplementary figure 3).

Taxonomic profiling of the Porites spp. endolithic community

The microbial community observed in the skeletons of *Porites* spp. was highly diverse and variable among samples within $p\mathrm{CO}_2$ conditions. Prokaryotic members of the microbiome (observed in the 16S rRNA gene dataset) accounted for most of the species diversity (Table 1), and no bacterial OTUs were present in all *Porites* spp. samples. The most abundant phylum recovered was *Proteobacteria*, followed by *Bacteroidetes* and Archaea (Figure 1A, Supplementary figure 2). The relative abundance of the nitrogen-fixing order *Rhizobiales* (Alphaproteobacteria) in all *Porites* spp. samples was 9.1% ± 4% standard deviation (hereafter ± only), and the phylum of green sulphur bacteria, *Chlorobi*, was 0.4% ± 1%. Members of the *Bacteroidetes* were twice as abundant at high $p\mathrm{CO}_2$ sites (12.1% ± 10% in reference versus 23.9% ± 17% in high $p\mathrm{CO}_2$ sites), mostly due to a higher abundance within the classes *Cytophagia* (4.8% ± 7% versus 10.4% ± 9%), *Flavobacteria* (2.2% ± 2% versus 3.7% ± 3%) and *Saprospirae* (2.4% ± 5% versus 9.4% ± 15%). We also observed a lower abundance of the Archaeal class *Parvarchaea* in the high $p\mathrm{CO}_2$ site (1.6% ± 0.9% versus 11.6% ± 12% in high $p\mathrm{CO}_2$ and reference sites, respectively). A representation of the relative abundances of bacterial phyla on a sample-by-sample basis can be found in Supplementary figure 2. These differences were, however, not statistically significant based on ANOVA and Kruskal-Wallis tests (Bonferroni-corrected *p*-values = 1, Supplementary tables S2 and S3).

The *tuf*A dataset (Figure 1B) suggested that the algal community was dominated (64.7% ± 36%) by lineages of the Ostreobiaceae (Chlorophyta, Bryopsidales). Although no *tuf*A OTUs were found to be omnipresent in *Porites* spp., siphonous green algae (order Bryopsidales) were present in all samples analysed here, indicating that they are ubiquitous members of the coral skeleton core microbiome. *Ostreobium* clade #1 showed the highest relative abundance (33% ± 40%), followed by *Ostreobium* clades #4, #3 and #2. While the relative abundance of clade #1 was similar between sites, the relative abundance of the other *Ostreobium* clades varied substantially, but not statistically significant, between reference and high $p\mathrm{CO}_2$ sites. A high abundance of an unclassified group of OTUs belonging to the green algal order Bryopsidales was also observed, particularly in the reference site (Figure 1B).

The prevalence of green algal lineages in the skeletons of *Porites* spp. was also suggested by the UPA dataset (Figure 1C), which shows that 86.8% ± 15% of the reads belong to the green algal order Bryopsidales. There are no UPA reference sequences for *Ostreobium* clades #1 and #2, therefore possible sequences of these clades might have been classified as clades #3, #4 or "unclassified Bryopsidales" by the RDP classifier, which may explain the differences in the abundances of *Ostreobium* clades between the *tuf*A and UPA datasets. One UPA OTU was found across all *Porites* spp. samples (OTU_3). This OTU was only classified at the kingdom level as a eukaryote (Supplementary table S2) and showed little similarity to known red algae and Stramenopiles species (blastn e-values ≤ 4e-79 but Identity ≤ 79%). Differences in the relative abundances between reference and high $p\text{CO}_2$ sites were minimal (Figure 1C, Supplementary figure 2).

Endolithic communities across different coral host species

The prokaryotic endolithic communities of *Seriatopora hystrix* and *Pocillopora damicornis* were significantly less rich than those found in *Porites* spp., as indicated by Chao1 and observed OTUs indices (*p*-value = 0.003, Table 2). A significant difference was detected in the relative abundances of certain OTUs belonging to the *Endozoicimonaceae* family between coral species, with the highest abundance in *P. damicornis* (Kruskal-Wallis, *p*-values < 0.0002, Supplementary table S4). The *Porites* spp. samples had a higher relative abundance of an OTU related to the order *Rhizobiales* (genus *Afifella*), and *P. damicornis* showed a significantly (*p*-value = 0.03) higher abundance of an OTU related to the phylum *Bacteroidetes* (order *Cytophagales*; Supplementary table S4). At higher taxonomic levels, the relative abundance of the phyla *Planctomycetes, Bacteroidetes*, and the bacterial phylum OD1 were significantly different among coral hosts (ANOVA, *p*-values = 0.007 and 0.01 respectively, Figure 3A, Supplementary figure 2, Supplementary table S5). Principal coordinate analysis (PCoA) showed evident differences in the prokaryotic microbiome associated with different coral hosts: *Porites* spp. samples clustered together, clearly separated from the two branching species (Figure 4A). The MGLM analysis confirmed a significant difference between the prokaryotic communities of different coral hosts (Figure 4A).

The alpha diversity of green algae (i.e. Chao 1 and Observed OTUs within the *tuf*A dataset) was significantly different between *S. hystrix* and *Porites* spp., but no significant differences were observed within the taxonomically broader spectrum of eukaryotic algae amplified with the UPA marker (Table 2). The Kruskal–Wallis test suggested no significant difference in the relative abundances of particular OTUs between host species (neither within the *tuf*A nor within the UPA dataset), at least when corrected (Bonferroni) *p*-values were taken into consideration (Supplementary table S4). After rarefying the sequences, the *tuf*A dataset was reduced to a single *P. damicornis* sample (Supplementary table S1), therefore we could not test for differences in alpha diversity or relative abundances in the *P. damicornis* community compared to other host corals. We observed a significantly different relative abundance of *Ostreobium* spp. (order Bryopsidales) among coral hosts within the UPA dataset (ANOVA, *p*-value = 0.000002), but not in the *tuf*A dataset (*p*-values > 0.05, Figure 3B and 3C, Supplementary figure 2, Supplementary table S5). *Seriatopora hystrix* had a high and variable (83% ± 57%) relative abundance of endolithic lineages related to the macroalga *Halimeda* spp. within the *tuf*A dataset, while this group constituted a minimal fraction (0.04% ± 0.2%) of the endolithic community of *Porites* spp. (Figure 3B). No pattern was observed within the PCoA plot of the community composition using the *tuf*A marker (Figure 4B). The UPA marker, which comprised more samples of the branching species, showed that *Porites* spp. samples cluster together and away from *S. hystrix* and *P. damicornis*, although two outliers belonging to branching samples cluster with *Porites* spp. (Figure 4C). The MGLM analysis suggested no significant differences between the algal communities of different coral hosts (Figure 4B and C), except when a presence-absence distance matrix was used (Supplementary materials, Supplementary figure 4).

Discussion

Our results show that the prokaryotic and eukaryotic microbiome in the skeletons of *Porites* spp. are highly diverse but indistinguishable between corals inhabiting naturally high $p\mathrm{CO}_2$ reefs and ambient conditions. Ocean acidification is predicted to affect the coral reef CaCO_3 budget and its biological associations (Meron *et al.* 2011; Andersson & Gledhill 2013; Morrow *et al.* 2015), and depending on the experiment, endolithic communities were shown to either exacerbate or buffer the effects of these environmental changes (Fine & Loya 2002; Tribollet *et al.* 2009; Reyes-Nivia *et al.* 2013). Our results suggest that the composition of endolithic communities, at least in *Porites* spp., is virtually unaffected by the surrounding high $p\mathrm{CO}_2$ water from a natural volcanic seep, and therefore less likely to be disturbed by OA than we previously thought. Although homogeneous between high $p\mathrm{CO}_2$ and reference sites, we show that the endolithic community is highly diverse and structured among coral host species.

A stable microbiome

The mechanisms influencing the structure of the endolithic microbiome (regardless of variable $p\mathrm{CO}_2$ conditions), are currently unknown. We raise here two hypotheses that may explain our results. The "stable habitat" hypothesis assumes that the endolithic environment is highly homeostatic so that pH is maintained inside the skeletons regardless of external changes in the surrounding water. The "tolerant endolith" hypothesis is based on the notion that endolithic microorganisms have a wide pH tolerance range, potentially wider than the microbes associated with the tissues and mucus.

The first hypothesis is supported by the ability of some corals to up-regulate the pH at the tissue-skeleton interface, which allows them to calcify and grow even under high $p\mathrm{CO}_2$ (McCulloch *et al.* 2012; Venn *et al.* 2013; Georgiou *et al.* 2015). The pH within coral cells remains relatively constant throughout the day (7.05-7.46 units), likely due to membrane transporters that extrude the excess of by-products of photosynthesis and respiration to maintain a stable intracellular pH (Laurent *et al.* 2013). This process may indirectly create a stable microhabitat within the coral skeleton that is protected from shifting pH in the surrounding seawater (see also Shashar *et al.* 1997). The observation that radioactivity in seawater impacted corals' living tissue but did not reach their endolithic zone (Odum & Odum 1955) supports this notion.

One problem with the stable habitat hypothesis is that the pH in the skeletons of *Porites* (*compressa*) can vary daily from 7.7 to 8.5 pH units, mostly due to the by-products of respiration and photosynthesis of the coral and *Symbiodinium* that are exported to the skeleton (Shashar & Stambler 1992). This daily variation is well above the projections of OA for the near future, which predicts a pH drop of 0.4 units by 2100, and up to 0.7 units by 2300 (Raven *et al.* 2005; Hoegh-Guldberg *et al.* 2007). A reasonable counterargument is that the direction of the movement (pH reduction due to OA) may be more important than the daily variation within skeletons – for example, microorganisms living under 7.7 to 8.5 pH units not necessarily withstand a seawater pH shift from 7.8 to 7.6 pH units. The resilience of at least some microbes might also be related to their boring mechanism, which involves a sophisticated control of intracellular pH (associated with calcium pumps and protons counter-transport) in endolithic cyanobacteria (Garcia-Pichel 2006; Garcia-Pichel *et al.* 2010). Therefore our second hypothesis that organisms exposed to daily pH fluctuations within the skeleton are adapted to cope with a wide range of $p\mathrm{CO}_2$ conditions may be more accurate. Experimental work and genomic data of endolithic organisms will help to test the tolerant endolith and the stable habitat hypotheses. For example, specialization to the low light experienced in the endolithic habitat has been observed in the plastid genome of *Ostreobium quekettii* (Marcelino *et al.* 2016) and the presumed pH tolerance may also be reflected in the genomes of endolithic organisms.

The lack of discernible differences in the endolithic community composition between high $p\mathrm{CO}_2$ and reference corals observed here is in agreement with the results of an experiment where *Balanophyllia europaea* and *Cladocora caespitose* corals were transplanted to a naturally high $p\mathrm{CO}_2$ area (Meron *et al.* 2012). In an aquarium-based experiment conducted over a shorter time, the bacterial community composition present in the tissue, skeleton and mucus of *Acropora eurystoma* were found to be affected by high $p\mathrm{CO}_2$, but further analysis using clone libraries suggested that only the prokaryotic communities of the mucus and tissue, not the skeleton, were affected by low pH (Meron *et al.* 2011). These different observations might be associated with the different time spans and experimental setups of the two studies, and it is likely that the microbial community associated with different coral taxa have different responses to acidification. The resilience of endolithic algae to acidification has also been observed: the net photosynthesis and respiration of algae growing at the surface of dead coral blocks was severely impacted upon exposure to high $p\mathrm{CO}_2$ treatments, while the endolithic flora was unaffected (Tribollet *et al.* 2006). Studies have demonstrated that endolithic algae actually benefit from low pH and tend to increase in biomass under high $p\mathrm{CO}_2$ conditions (Tribollet *et al.* 2009; Reyes-Nivia *et al.* 2013; Enochs *et al.* 2016).

The observation that high $p\mathrm{CO}_2$ did not impact the endolithic community of *Porites* spp. does not necessarily imply that coral holobionts are immune to ocean acidification. It is possible that the methods used here are not sufficiently powerful to detect the effects of high $p\mathrm{CO}_2$ on endolithic microbial communities. However, the fact that we detected significant differences among coral hosts even though the sampling size for the branching corals was smaller, indicates that our methods and sampling design are adequate and it is unlikely that differences among high $p\mathrm{CO}_2$ and reference sites were present but went undetected. It is possible though that high $p\mathrm{CO}_2$ impacts the endolithic communities of other coral species that were not examined. It is interesting to note that massive *Porites* spp. dominate the reef near volcanic seeps while the presence of branching species (e.g. *Acropora* spp.) was largely reduced (Fabricius *et al.* 2011). Our analyses are restricted to the volcanic seeps of Milne Bay, which have relatively small areas under high $p\mathrm{CO}_2$ and are surrounded by ambient seawater. Further studies at additional sites impacted by high $p\mathrm{CO}_2$ and across a wider range of coral species is required to evaluate the results in our study across the broader ecological context of effects of OA on coral microbiomes.

Diversity and potential functional roles of the endolithic microbiome

Bacteria related to *Endozoicomonas* spp. (class Gammaproteobacteria) are predicted to have a key role in the coral holobiont. These bacteria have been shown to be endosymbionts, forming aggregations within coral tissues (Neave *et al.* 2017), potentially contributing to nutrient cycling and structuring of the microbiome through the production of quorum-sensing signalling metabolites and antimicrobial compounds (Meyer *et al.* 2014; Morrow *et al.* 2015 and references therein). The relative abundance of *Endozoicomoniaceae* within coral tissues appears to be sensitive to high $p\mathrm{CO}_2$ (Morrow *et al.* 2015; Webster *et al.* 2016), but in the skeletons of *Porites* spp. analysed here, they did not differ significantly between samples from different $p\mathrm{CO}_2$ conditions (Supplementary Table S2). We observed a significantly higher relative abundance of two *Endozoicomoniaceae* OTUs in the skeletons of *P. damicornis* when compared to the other two coral species, possibly reflecting stable associations of *Endozoicomoniaceae* species with this coral host (see Neave *et al.* 2017). Although some of the sequences retrieved here may derive from other parts of the coral, other studies have also detected members of *Endozoicomonaceae* in the endolithic community (Williams *et al.* 2015; Marcelino & Verbruggen 2016; see also Ainsworth *et al.* 2015).

Bacteria in the phylum *Bacteroidetes* are often associated with coral disease and have been shown to increase in relative abundance under reduced pH (Vega Thurber *et al.* 2009). The average relative abundance of this group doubled in endolithic communities from high $p\mathrm{CO}_2$ sites, but this difference was not significant likely due to the high level of variation in community composition among colonies within sites. This increase was mostly due to a higher abundance of the classes *Saprospirae*, *Flavobacteria* and *Cytophagia*, which contain most of the known marine algicide bacteria (Furusawa *et al.* 2003; Mayali & Azam 2004; Zozaya-Valdes *et al.* 2015). It is plausible that a higher relative abundance of these microorganisms is associated with an increase in endolithic algal biomass under high $p\mathrm{CO}_2$ (see Reyes-Nivia *et al.* 2013, Johnson *et al.* 2017). Rather than compromising coral health, these bacteria may control excessive endolithic algal growth and may help to maintain a stable community composition under ocean acidification.

Microorganisms involved in nitrogen cycling may be fundamental to coral resilience to ocean acidification and climate change (Rädecker *et al.* 2014; Santos *et al.* 2014; Radecker *et al.* 2015). We found a diverse community of nitrogen fixing (diazotrophic) microorganisms inhabiting coral skeletons. The majority (in terms of relative abundances) belonged to the order *Rhizobiales*, a group that appears to form stable symbiotic associations with corals (Lema *et al.* 2014). Green sulphur (also diazotrophic) bacteria in the phylum *Chlorobi*, previously documented as prevalent members of the endolithic community in the coral *Isopora* (Yang *et al.* 2016), were found at low relative abundances in the samples analysed here and in a previous study (Marcelino & Verbruggen 2016). Cyanobacterial OTUs captured with the UPA marker, while not abundant, were very diverse and mostly unclassified at lower taxonomic levels. Cyanobacteria have been shown to fix nitrogen in coral tissues (Lesser *et al.* 2004; Radecker *et al.* 2015) and can be responsible for a large fraction of the nitrogen fixation observed in their skeletons (Crossland & Barnes 1976; Davey *et al.* 2007).

Endolithic algal biomass has been shown to increase under high $p\mathrm{CO}_2$, as phototrophic organisms benefit from the increased availability of carbon dioxide for photosynthesis (Tribollet *et al.* 2009; Reyes-Nivia *et al.* 2013). Indeed, we observed a higher relative abundance of all *Ostreobium* clades in *Porites* spp. samples from high $p\mathrm{CO}_2$ sites, but the variability among replicates (i.e. *Porites spp.* samples within sites) was also high, making it difficult to draw conclusions about whether *Ostreobium* spp. are competitively superior to other endolithic algal lineages under OA. Whether the increase in algal biomass is a threat to corals under OA depends on whether the associated bioerosion levels will exceed reef accretion (calcification). Besides increasing bioerosion, excessive endolithic algal growth can penetrate the coral living tissue, possibly increasing their susceptibility to infections (Peters 1984; Fine *et al.* 2006). An increase in endolithic algae may also be beneficial to the coral by providing them with vital nutrients, which is especially important during coral bleaching events (Schlichter *et al.* 1995; Fine & Loya 2002).

The possibility that the endolithic microbiome contributes to the resilience of corals under future OA conditions deserves further attention. Massive *Porites* spp. may be considered more competitive under OA than branching species based on their prevalence at naturally high $p\mathrm{CO}_2$ sites (Fabricius *et al.* 2011). We visibly observed that our massive *Porites* spp. samples had higher colonisation with endolithic algae compared to the branching species. The photosynthetic activity of *Symbiodinium* plays an important role in maintaining pH homeostasis within corals (Gibbin *et al.* 2014), and it is possible that endolithic algae provide a similar service within the skeleton. The biomass of endolithic algae may exceed that of *Symbiodinium* by 16-fold (Odum & Odum 1955) and can contribute significantly to the buffering capacity of the holobiont (see Yamazaki *et al.* 2008; Reyes-Nivia *et al.* 2014). It is noteworthy that several functionally important microorganisms (e.g. *Endozoicomoniaceae* and *Bacteroidetes*) often found in coral tissues and mucus also occur in coral skeletons (Sweet *et al.* 2010; Ainsworth *et al.* 2015; Willians *et al.* 2015; Marcelino & Verbruggen 2016; this study). It is possible that some of these microorganisms were initially associated with polyp tissues, but remained after removing the tissues from the skeleton with pressurized air, especially in *Porites* spp. which is a perforate coral with tissues that penetrate the skeleton, or that they penetrated the coral skeleton when the samples were placed in the storage buffer. It is noteworthy however that multiple studies have found tissue-associated microbes within coral skeletons, and that the majority of OTUs commonly found in healthy corals have also been found in bare coral skeleton, but not in seawater or in a diseased coral tissue (Fernando *et al.* 2015). It is possible therefore that the coral skeleton serves as a reservoir for the microbiome and provides a source of beneficial bacteria to coral tissues, analogous to the human appendix which functions as a safe house for symbiotic microbes that repopulate the intestine following acute illness (Randal Bollinger *et al.* 2007). Acute environmental stress can disrupt symbiotic relationships among hosts and symbionts (see Hawkins *et al.* 2013), and a stable endolithic community may assist in the recovery of the coral microbiome after environmental (and/or physiological) conditions stabilize.

Different host species harbour distinct endolithic communities

The endolithic communities of the branching corals *Seriatopora hystrix*, *Pocillopora damicornis* and the massive *Porites* spp. contain significantly different relative abundances of functionally important members of the microbiome (including species of *Endozoicimonaceae* and *Bacteroidetes*) and appear to separate based on morphology or taxonomy (as both branching species belong to the family *Pocilloporidae*). First, the two branching species harbour a reduced diversity of bacteria and algae. The low relative abundance of *Ostreobium* spp. in the endolithic communities of branching species is surprising, considering the generally ubiquitous nature of this alga in coral skeletons (Odum & Odum 1955; Tribollet 2008a; Gutner-Hoch & Fine 2011). Instead of *Ostreobium* spp., the coral *S. hystrix* has a high relative abundance of OTUs related to a macroalga (*Halimeda* spp.), which has only recently been reported in coral skeletons. It is possible that *Halimeda* spp. occur in the coral skeleton in the form of rhizoids that have penetrated the limestone, or most likely, as an unknown microscopic and endolithic life stage of two *Halimeda* species (*H. discoidea* and *H. micronesica*) that are commonly present in metabarcoding studies of endolithic communities (Marcelino & Verbruggen 2016; Sauvage *et al.* 2016; this study).

The observed differences in endolithic community composition among coral hosts may be a result of specialization to particular host traits or reflect co-evolution between coral hosts and endolithic species. The living tissue of *Porites* (*lobata*) is about five times thicker, penetrates the skeleton and contains a higher density of *Symbiodinium* than the living tissue of *P. damicornis* and *S. hystrix* (Yost et al. 2013). Tissue thickness would influence the amount of light that penetrates and reflects within the inner parts of the skeleton and may influence the composition of the endolithic community. Branching coral species also tend to grow faster than massive corals (Gates & Ainsworth 2011), and the branch tips collected in this study may have a younger population of endoliths in comparison to more mature sections of the colony base (a pattern reported in Pica *et al.* 2016). Future studies would benefit from examining the microbiome associated with different areas of the colony and possible specialization to skeletal features. Alternatively (and not mutually exclusively), endolithic lineages might form stable community assemblies that have co-evolved with the coral host, or the host species have some control over the composition of the endolithic community by selecting beneficial taxa. Mutualistic relationships between corals and their endolithic associates have been suggested in several studies (Odum & Odum 1955; Schlichter *et al.* 1995; Schlichter *et al.* 1997; Fine & Loya 2002; Försterra & Häussermann 2008; Titlyanov *et al.* 2009), and future research would benefit from characterizing possible co-evolutionary processes among coral species and endolithic microorganisms.

Conclusions

This study reports a diverse microbiome within the skeletons of *Porites* spp., and demonstrates that little discernible patterns exist in this microbiome across ambient and naturally high $p\mathrm{CO}_2$ environments. We show that the endolithic community shares several functionally important microbes with the coral tissue layer. Environmental stress can induce corals to lose their symbiotic microorganisms, and a diverse endolithic microbial community might serve as a reservoir to recolonise the microbiome in the coral tissue after the re-establishment of their physiological equilibrium. We found functionally important members in the endolithic community, including members in the *Endozoicimonaceae* and *Bacteroidetes*, forming distinct associations with the different host coral families, an observation consistent with the endolithic reservoir proposition. The diversity and community structure observed in this study form the baseline for future studies aiming to investigate the roles of endolithic microorganisms in enabling corals to endure climate change.

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Data Accessibility

DNA sequences have been deposited in NCBI's Sequence Read Archive (SRA) under the accession IDs SAMN07251731 – SAMN07251770.

Author Contributions

KMM and DGB conceived and conducted the sampling. VRM performed DNA extractions, library preparation, analyses and drafted the manuscript. MvO, KMM, DGB and HV reviewed the analyses and contributed to writing.

Tables:

Table 1: Diversity indices based on the microbiome of *Porites* spp. skeletons from reference and high $p\mathrm{CO}_2$ sites and standard deviations (±). Chao1 and Number of Observed OTUs between high $p\mathrm{CO}_2$ and reference sites were compared with a two-sample t-test, p-values ≤ 0.05 suggest significant differences between sites, but were not observed. N = number of samples after rarefaction. Seqs = rarefaction threshold. OTUs = number of OTUs retrieved in each dataset after quality filtering.

				Chao1			**Obs. OTUS**		
	N	**Seqs**	**OTUs**	**Reference**	**high $p\mathrm{CO}_2$**	***p*-value**	**Reference**	**high $p\mathrm{CO}_2$**	***p*-value**
16S	20	2200	890	141.2 ± 52.4	148.5 ± 45.8	0.79	133.6 ± 49.9	140.7 ±44.8	0.77
***tuf*A**	22	1400	59	7.8 ± 3.3	7.4 ± 2.8	0.78	7.7 ± 3.2	7.3 ± 2.8	0.74
UPA	22	7000	164	21.6 ± 7.1	24.4 ± 5.4	0.33	20.6 ± 6.7	23.2 ± 5.1	0.33

16S rRNA gene Chao1

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	*p*-value
S. hystrix	*P. damicornis*	29.087	29.494	21.514	15.705	0.507	1.000
S. hystrix	*Porites* spp.	29.087	29.494	125.296	49.889	-4.363	0.003*
P. damicornis	*Porites* spp.	21.514	15.705	125.296	49.889	-4.854	0.003*

16S rRNA gene observed OTUs

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	*p*-value
S. hystrix	*P. damicornis*	21.767	19.758	19.083	12.331	0.258	1.000
S. hystrix	*Porites* spp.	21.767	19.758	101.274	37.282	-4.865	0.003*
P. damicornis	*Porites* spp.	19.083	12.331	101.274	37.282	-5.138	0.003*

***tuf*A Chao1**

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	*p*-value
S. hystrix	*P. damicornis*	2.033	0.858	3.450	0.000	1.168	1.000
S. hystrix	*Porites* spp.	2.033	0.858	9.606	3.849	-3.264	0.006*
P. damicornis[1]	*Porites* spp.	3.450	0.000	9.606	3.849	-1.531	0.309[1]

***tuf*A observed OTUs**

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	*p*-value
S. hystrix	*P. damicornis*	1.933	0.736	3.000	0.000	1.024	1.000
S. hystrix	*Porites* spp.	1.933	0.736	9.330	3.623	-3.388	0.003*
P. damicornis[1]	*Porites* spp.	3.000	0.000	9.330	3.623	-1.673	0.192[1]

UPA Chao 1

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	*p*-value
S. hystrix	*P. damicornis*	19.353	14.158	13.386	16.038	0.624	1.000
S. hystrix	*Porites* spp.	19.353	14.158	21.732	6.187	-0.591	1.000
P. damicornis	*Porites* spp.	13.386	16.038	21.732	6.187	-1.921	0.162

UPA observed OTUs

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	*p*-value
S. hystrix	*P. damicornis*	18.100	12.882	12.567	14.816	0.630	1.000
S. hystrix	*Porites* spp.	18.100	12.882	19.539	5.572	-0.394	1.000
P. damicornis	*Porites* spp.	12.567	14.816	19.539	5.572	-1.754	0.327

Table 2: Comparison of endolithic community diversity indices between *Porites* spp., *Seriatopora hystrix* and *Pocillopora damicornis* corals. Chao1 and Number of observed OTUs were compared with a two-sample t-test, *p*-values ≤ 0.05 indicate significant differences between host species and are marked with an asterisk. Std = standard deviations.

1 - The *tuf*A dataset has only one *P. damicornis* sample (after rarefaction), therefore the significance cannot be calculated. See Supplementary table S1 for number of samples.

Figure 1

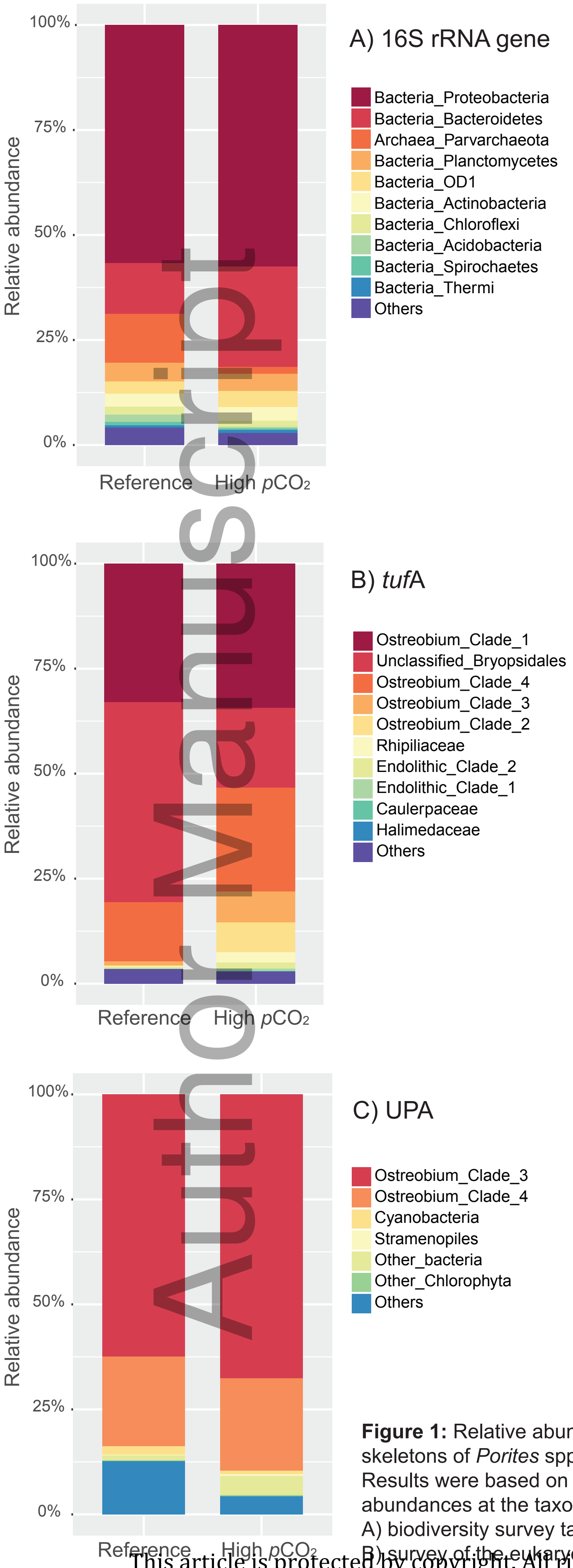

Figure 1: Relative abundances of the most common microbial taxa in coral skeletons of *Porites* spp. from high pCO_2 and reference sites.
Results were based on all samples from each site, averaging the relative abundances at the taxonomic level displayed in the legend.
A) biodiversity survey targeting prokaryotes based on the 16S rRNA gene;
B) survey of the eukaryotic green algal members of the microbiome based on the *tuf*A marker; C) biodiversity survey using the Universal Plastid Amplicon.

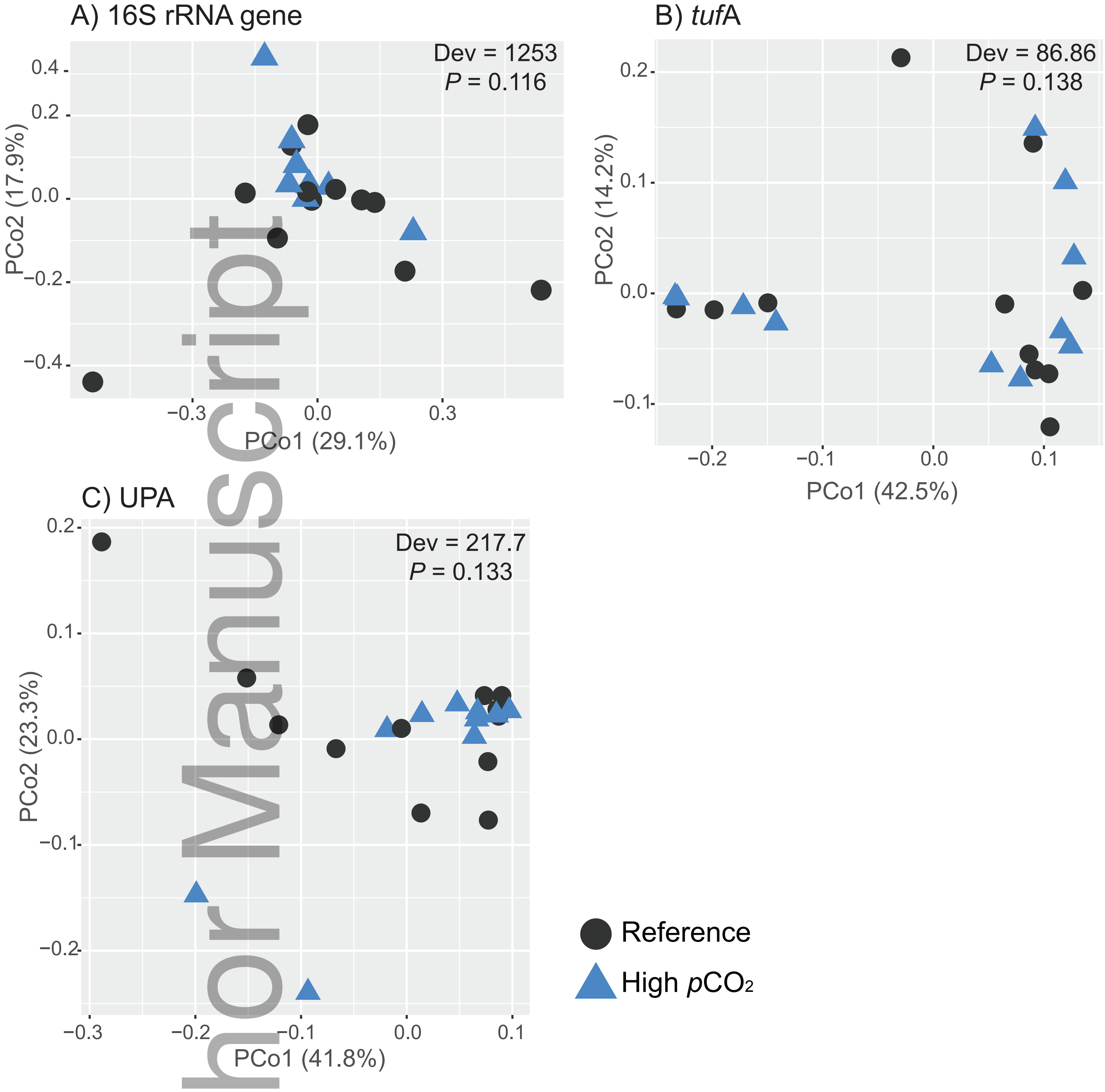

Figure 2: Principal Coordinate Analysis of microbial communities present in limestone skeletons of *Porites* spp. from high pCO_2 and reference sites. The analyses were based on weighted UniFrac distance matrices calculated with OTU-level abundances for each metabarcoding marker: A) prokaryotic 16S rRNA gene marker; B) eukaryotic green algae *tuf*A marker; C) Universal Plastid Amplicon marker. The results of the MGLM analysis (Deviance and *P* value) are shown.

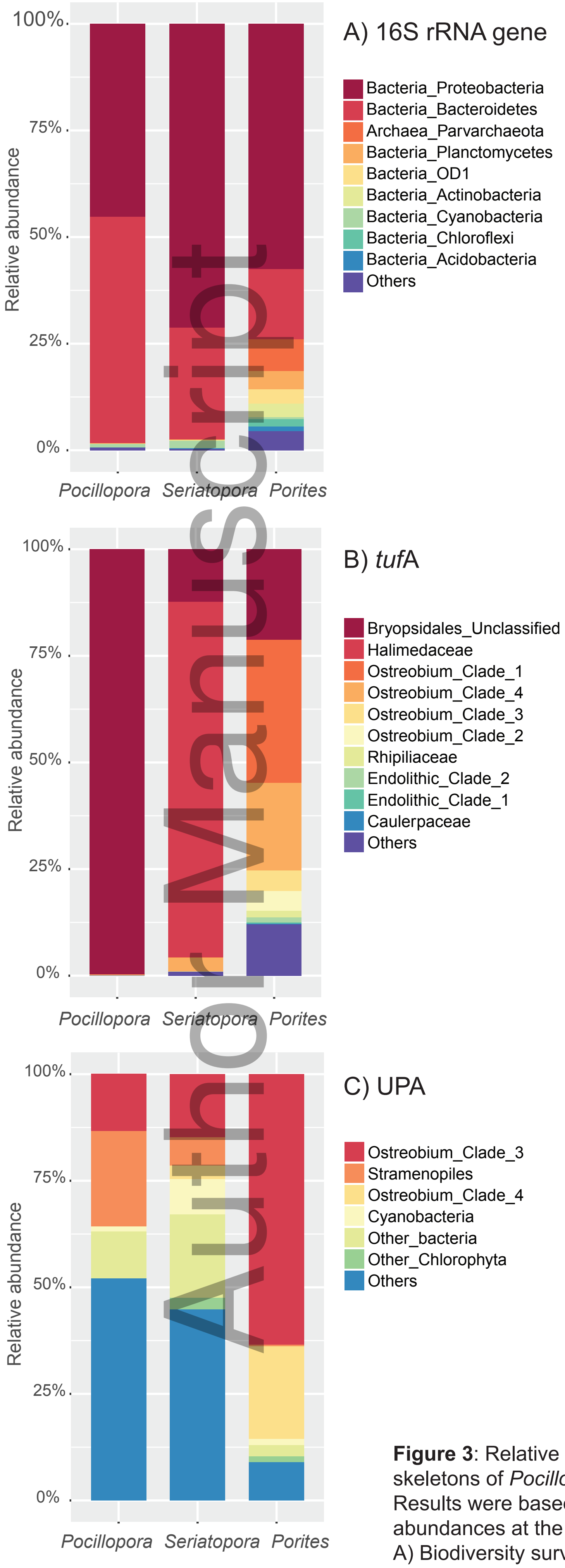

Figure 3: Relative abundances of the most common microbial taxa in coral skeletons of *Pocillopora damicornis*, *Seriatopora hystrix* and *Porites* spp. Results were based on all samples from each coral species, averaging the relative abundances at the taxonomic level displayed in the legend. A) Biodiversity survey targeting prokaryotes based on the 16S rRNA gene; B) survey of the eukaryotic green algal members of the microbiome based on the *tuf*A marker; C) biodiversity survey using the Universal Plastid Amplicon

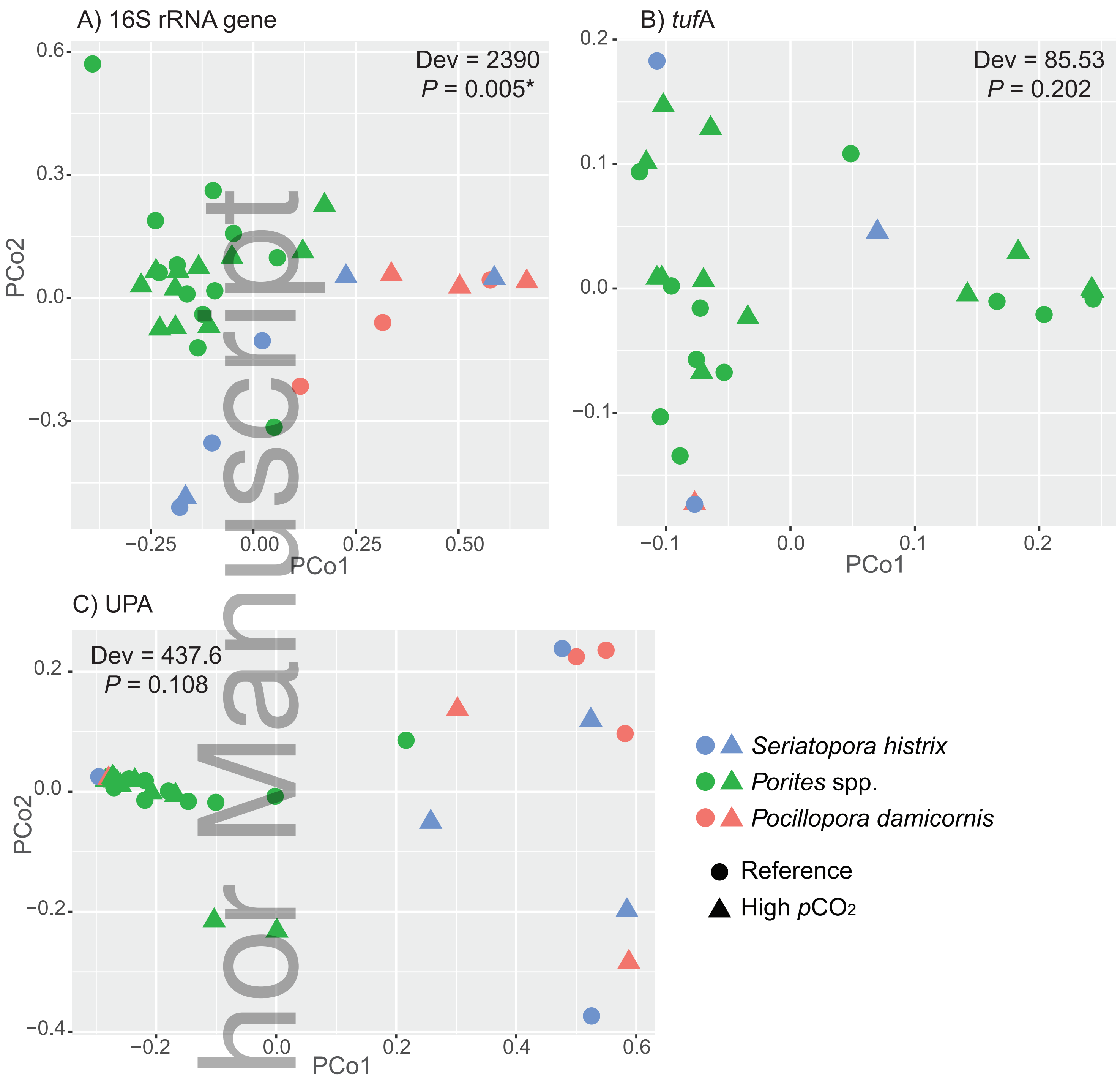

Figure 4. Principal Coordinate Analysis of microbial communities present in limestone skeletons of three coral host species collected in high $p\mathrm{CO}_2$ and reference sites. The analyses were based on weighted UniFrac distance matrices calculated with OTU-level abundances for each metabarcoding marker: A) prokaryotic 16S rRNA gene marker; B) eukaryotic green algae *tuf*A marker; C) Universal Plastid Amplicon marker. The results of the MGLM analysis (Deviance and P value) are shown.