# Coral tissue and mucus microbiomes differ in their sensitivity to host and environmental variation

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Corals associate with diverse microbial assemblages; however, the spatial-temporal dynamics of intra-species microbial interactions are poorly understood. The coral-associated microbial community varies substantially between tissue and mucus microhabitats, however the factors controlling the occurrence, abundance and distribution of microbial taxa over time have rarely been explored for different compartments simultaneously. Here, we assess the effect of host and environmental factors on microbiome communities in different coral compartments (surface mucus and tissue) of two *Acropora* species (*A. tenuis* and *A. millepora*) common along inshore reefs of the Great Barrier Reef. Amplicon based 16S ribosomal RNA gene sequencing of 136 samples collected over 14 months, revealed significant differences in bacterial richness, diversity and community structure among mucus, tissue and the surrounding seawater. Seawater samples were dominated by members of the Synechococcaceae and Pelagibacteraceae bacterial families whereas the mucus microbiome of *Acropora* spp. was dominated by members of Flavobacteriaceae, Synechococcaceae and Rhodobacteraceae and the tissue was dominated by Endozoicimonaceae. Environmental factors including levels of chlorophyll *a,* ammonium, particulate organic carbon and the sum of nitrate and nitrite were the primary drivers of the mucus microbiome in both *Acropora* species. In contrast, the response of the tissue microbiome to environmental and physiological factors differed between host species, suggesting host-specific modulation of the environmental drivers of tissue-associated microbiome. These results highlight that microbiomes inhabiting different compartments within the coral holobiont differ in their response to host physiology and environmental factors.

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## Abstract

Corals associate with diverse microbial assemblages; however, the spatial-temporal dynamics of intra-species microbial interactions are poorly understood. The coral-associated microbial community varies substantially between tissue and mucus microhabitats, however the factors controlling the occurrence, abundance and distribution of microbial taxa over time have rarely been explored for different compartments simultaneously. Here, we assess the effect of host and environmental factors on microbiome communities in different coral compartments (surface mucus and tissue) of two *Acropora* species (*A. tenuis* and *A. millepora*) common along inshore reefs of the Great Barrier Reef. Amplicon based 16S ribosomal RNA gene sequencing of 136 samples collected over 14 months, revealed significant differences in bacterial richness, diversity and community structure among mucus, tissue and the surrounding seawater. Seawater samples were dominated by members of the Synechococcaceae and Pelagibacteraceae bacterial families whereas the mucus microbiome of *Acropora* spp. was dominated by members of Flavobacteriaceae, Synechococcaceae and Rhodobacteraceae and the tissue was dominated by Endozoicimonaceae. Environmental factors including levels of chlorophyll *a,* ammonium, particulate organic carbon and the sum of nitrate and nitrite were the primary drivers of the mucus microbiome in both *Acropora* species. In contrast, the response of the tissue microbiome to environmental and physiological factors differed between host species, suggesting host-specific modulation of the environmental drivers of tissue-associated microbiome. These results highlight that microbiomes inhabiting different compartments within the coral holobiont differ in their response to host physiology and environmental factors.


## Introduction

Corals associate with diverse microbial assemblages including the well-characterized endosymbiotic association with eukaryotic dinoflagellates in the family Symbiodiniaceae*,* and a diversity of prokaryotes (Bacteria and Archaea), generally referred to as the coral microbiome (Bourne, Morrow & Webster, 2016; Frade et al., 2016a; Rowher et al., 2002). The microbiome has a fundamental role in the health and stability of the coral holobiont; it recycles nutrients, removes waste products and can act in defense against pathogens (Lema, Willis & Bourne, 2012; Morris et al., 2011; Rädecker et al., 2015; Rosado et al., 2019). The composition of the coral microbiome can be influenced by a variety of intrinsic and extrinsic factors. Coral microbiomes

were described to be host species-specific and to remain relatively stable over space and time (Frias-Lopez et al., 2002; Rowher et al., 2002). However, recent studies have proposed that spatial-temporal factors such as environmental parameters (Chen et al., 2011), depth (Glasl et al., 2017), geography (Hong et al., 2009; Littman et al., 2009a), seasonality (Ceh, van Keulen & Bourne, 2011; Chen et al., 2011; Hong et al., 2009; Koren & Rosenberg, 2006), coastal pollution (Klaus et al., 2007), and the physiological status of the host (Grottoli et al., 2018; Littman, Willis & Bourne, 2009b) can also influence the occurrence and relative abundance of microbial taxa. For instance, Li et al. (2015) reported a dynamic relationship between the community structure of coral-associated bacteria and the seasonal variation in environmental parameters such as dissolved oxygen and rainfall. Glasl et al. (2019a) showed that although host-associated microbiomes were five-times less responsive to the environment as compared to the seawater microbiome, they were still affected by environmental factors (e.g. temperature, turbidity, and nutrient concentration).

The coral animal encompasses different microhabitats for its microbial associates, including the surface mucus layer, coral tissue, skeleton and gastrovascular cavity (Agostini et al., 2012; Engelen et al., 2018; Pollock et al., 2018; Sweet, Croquer & Bythell, 2011). Each microhabitat has a unique set of biochemical features and harbors a specific microbial community (Engelen et al., 2018; Pollock et al., 2018; Sweet, Croquer & Bythell, 2011). Hence, revealing microhabitat-specific host-microbiome associations and their sensitivities to environmental fluctuations is crucial to our understanding of coral symbiosis. For example, the coral surface mucus layer is a polysaccharide-protein-lipid complex that provides an interface between the coral epithelium and the surrounding seawater (Brown & Bythell, 2005). Here microbes take advantage of a nutrient-rich medium and particular microbiome members found in the coral mucus overlap with both the tissue and the seawater microbial communities (Bourne & Munn, 2005; Brown & Bythell, 2005; Glasl, Herndl & Frade, 2016; Sweet, Croquer & Bythell, 2011). In contrast to the extracellular polymeric nature of surface mucus layer, the coral tissue consists of two distinct layers (epidermis and gastrodermis) and a connective-tissue layer, the mesoglea (Muller-Parker, D'Elia & Cook, 2015). The coral tissue hosts photosymbiotic dinoflagellates (family Symbiodiniaceae), that can provide up to 100% of energy required by their coral host (Muller-Parker, D'Elia & Cook, 2015). Symbiodiniaceae communities have been shown to vary in tandem with bacterial communities in early life stages of corals (Quigley et al.,

2019) and this may be caused by the release of complex organic molecules such as the organosulfur compound dimethylsulfoniopropionate (DMSP; Bourne et al., 2013; Frade et al., 2016b). The coral tissue microbiome is mostly represented by bacteria belonging to the phyla Proteobacteria and Actinobacteria. For example, the gammaproteobacterial *Endozoicomonas* are abundant in the coral's endodermal tissue and are often considered 'true' coral symbionts (Bayer et al., 2013; Glasl et al., 2019b; Neave et al., 2016, 2017). When compared to the surface mucus layer, the prokaryotic community in the tissue is significantly less dense and diverse (Bourne & Munn, 2005; Koren & Rosenberg, 2006), likely attributed to the more spatially stable and host controlled environment (Bourne & Munn, 2005). Furthermore, tissue-associated bacterial communities form aggregations within the coral cell layers, also referred to as coral-associated microbial aggregates (CAMAs), and are often co-localized near Symbiodiniaceae cells highlighting potential metabolic interactions between symbionts (Wada et al., 2019).

In this study, we test the hypothesis that different coral compartments (surface mucus layer and tissue) of *Acropora* spp. harbor distinct microbial communities and that microbiome dynamics within these compartments are driven by different factors. We hypothesize that the mucus-associated microbial community is primarily influenced by environmental parameters due to its direct contact to the surrounding seawater environment, while the tissue-associated microbial community responds more to changes in the physiology of the host.

## Materials & Methods

### Sample collection

Samples of *Acropora millepora* (Ehrenberg, 1834)*, Acropora tenuis* (Dana, 1846) and seawater were collected monthly, at Geoffrey Bay (Magnetic Island) in the Great Barrier Reef (Supplementary Figure S1), between February 2016 and March 2017, for amplicon based 16S ribosomal RNA (rRNA) gene sequencing along with environmental metadata. The period was divided in two seasons: from May to October, classified as dry or winter season, and from November to April as wet or summer season according to the Bureau of Meteorology of the Australian Government (Commonwealth of Australia, 2019). All samples were collected under the permit G16/38348.1 issued by the Great Barrier Reef Marine Park Authority.

Samples (n = 3 per sample type and per sampling event) for molecular analysis were collected as part of the Australian Microbiome Initiative and the sample procedure has

previously been outlined by Glasl et al. (2019a). In brief, coral nubbins of both *Acropora* species were collected, rinsed with 0.2 µm filter-sterilized seawater and placed into cryogenic vials. Coral mucus from the same specimens was collected with sterile cotton swabs as previously described by Glasl, Herndl & Frade (2016). Seawater samples for molecular analysis were collected in sterile collapsible bags, pre-filtered through a 50 µm filter mesh to remove large particles, and subsequently filtered onto a 0.2 µm Sterivex filter (Millipore). All samples were immediately snap frozen in liquid nitrogen after collection and stored at -80°C until further processing. To acquire environmental information, water and sediment samples were collected in duplicate for each sampling event and further analyzed according to the standard procedures of Australian Institute of Marine Science (AIMS) to determine salinity, particulate organic carbon, total suspended solids, concentrations of chlorophyll *a,* ammonium, the sum of nitrite and nitrate, particulate nitrogen, nitrite, total nitrogen, non-purgeable organic carbon, non-purgeable inorganic carbon, phosphate, silica, total organic carbon in the sediment, total organic nitrogen in the sediment and grainsize percentage of sediments < 0.63 µm, between 0.63 µm and 2 mm, and > 2 mm (Devlin & Lourey, 2000; Glasl et al., 2019a). Seawater temperatures and daylight hours were obtained from AIMS long-term monitoring temperature records (http://eatlas.org.au).

**Sample preparation and genetic assays**

Frozen coral tissue was airbrushed into a *ziploc* bag with phosphate-buffered saline (PBS) solution added until all tissue was removed from the skeletal fragment (PBS volume noted). The resulting tissue slurry was homogenized for 1 min at 12,500 rpm using a hand-held tissue homogenizer (Heidolph Silent Crusher M), pelleted (10 min at 16,000 rcf) and snap frozen in liquid nitrogen. DNA from the tissue and mucus samples was extracted using the DNeasy PowerBiofilm kit (QIAGEN). DNA extracts were sent on dry ice to the Ramaciotti Centre for Genomics (Sydney, Australia) for sequencing. The bacterial 16S rRNA gene was sequenced using the 27F (Lane, 1991) and 519R (Turner et al., 1999) primers on the Illumina MiSeq platform using a dual indexed 2 x 300 bp paired-end approach.

**Sequence analysis**

Sequencing data were analyzed as single nucleotide variants following the standardized platform of the Australian Microbiome Initiative (Brown et al., 2018). In brief, paired-end reads were

merged using FLASH software (Magoc & Salzberg, 2011) and FASTA formatted sequences were extracted from FASTQ files. Sequences < 400 bp in length and / or containing N's or homopolymer runs of > 8 bp were removed with MOTHUR (v1.34.1; Schloss et al., 2009). Sequences were de-replicated and ordered by abundance using USEARCH (64 bit v10.0.240; Edgar, 2010). Sequences with less than 4 representatives and Chimeras were removed, and the quality-filtered sequences were mapped to chimera-free zero-radius operational taxonomic units (zOTUs). A table containing the samples and their read abundances was created and the zOTUs were taxonomically classified with SILVA v132 database (Yilmaz et al., 2014) using MOTHUR's implementation of the Wang classifier (Wang et al., 2007) and a 60% Bayesian probability cut-off. This sequencing dataset has already been used in a previous contribution by the research group (Glasl et al., 2019a), but in the current study it is analyzed from a different perspective aiming at comparing temporal microbiome dynamics between two distinct coral compartments.

Chloroplasts and mitochondria derived reads were removed from the dataset and remaining data was rarefied to a sequencing depth of 3,500 reads per sample in R (R Core Team, 2015) using subset_taxa () function in the phyloseq package (McMurdie & Holmes, 2013). The counts of reads per sample was transformed into relative abundances.

**Photopigment quantification**

Photopigment (chlorophyll *a*) concentrations were quantified using a spectrophotometric approach. Tissue pellets were thawed on ice to avoid sample degradation and resuspended in 1 ml of 90% ethanol. Samples were sonicated for 1 min and centrifuged for 5 min at 10,000 rcf. Subsequently, 700 µl of the supernatant was removed and transferred to a new tube. The resuspension, sonication and centrifugation were repeated on the remainder of the pellet. The supernatant was recovered again, combined with the previous extraction and mixed by inversion. Sample extract and 90% ethanol (blank read) were loaded in triplicate (200 µl each) to a 96-well plate and the absorbance was recorded at 470, 632, 649, 665, 696 and 750 nm in a Cytation 3 multi-mode microplate reader (BioTek, Winooski, USA) and analyzed using the software Gen5 (BioTek, Winooski, USA). Blank corrected absorbance measures were used to calculate chlorophyll *a* concentrations (Supplementary Equation S1).

**Protein quantification**

Protein concentrations of coral tissue samples were quantified using a colorimetric protein assay kit (Pierce BCA Protein Assay Kit). Tissue pellets were thawed on ice and resuspended in 1 ml PBS. The resuspension (25 µl) was added to 200 µl of working reagent from the kit in a 96-well plate. The plate was mixed thoroughly on a plate shaker for 30 seconds and then incubated at 37°C for 30 min. The plate was cooled down at room temperature. The absorbance was measured at 563 nm in a Cytation 3 multi-mode microplate reader (BioTek, Winooski, USA) and analyzed using the software Gen5 (BioTek, Winooski, USA). The measurements of the standards and samples were blank corrected to remove background absorbance. For each plate, a protein standard curve was obtained using bovine serum albumin (BSA) solution at concentrations between 25 and 2,000 µg $ml^{-1}$. Total protein concentration within each sample was calculated using the standard curve.

**Symbiodiniaceae cell counting**

To determine cell numbers of Symbiodiniaceae in the coral tissue, the tissue pellet was thawed on ice, resuspended in 1 ml of 0.2 µl filtered seawater and added to 1 ml of 10 % formalin to preserve the symbiont cells. The solution was passed through a syringe needle to reduce cell agglomeration and diminish the bias from cell clumps. Samples were then mixed for 1 min and 10 µl of the homogenate was loaded onto a Neubauer haemocytometer (0.100 mm depth). Symbiodiniaceae cells were counted under 40 x magnification with an Olympus CX31 light microscope. In total, 24 haemocytometer squares (each with 0.1 µl volume) were used per sample to ensure robustness of density determinations.

**Statistical analyses**

Statistical analyses were performed using RStudio (v1.1.463). Alpha- and beta-diversity analysis of microbial communities were performed on relative abundance data at zOTU level. zOTU richness and Shannon-Weaver diversity were compared across host compartments, host species and reference seawater samples using Analysis of Variance (ANOVA). A Venn diagram was performed to describe the shared and unique OTUs among mucus, tissue and seawater microbiomes using VennDiagram package (Chen & Boutros, 2011). Non-Metric Multidimensional Scaling (NMDS) was used to illustrate the microbial community structure

among host species and host compartments based on Bray-Curtis dissimilarities (phyloseq package, McMurdie & Holmes, 2013). Permutational Multivariate Analysis of Variance (PERMANOVA, 999 permutations) was used to test for differences in microbial structure among host species, host compartments and season using the adonis2() function of the vegan package (Oksanen et al., 2013).

Measurements of coral surface area were unavailable, hence physiological variables were normalized (i.e., chlorophyll *a* normalized to protein content, chlorophyll *a* normalized to Symbiodiniaceae numbers, Symbiodiniaceae density normalized to protein content) following common procedures in coral physiology studies (Frade et al., 2008). Environmental and physiological variables were standardized and checked for collinearity using the Pearson correlation coefficient. Redundant variables based on Pearson's correlation ($> 0.7$ or $< -0.7$; Dormann et al., 2013) were removed from the analysis. For environmental samples, Principal Component Analysis (PCA) was used to check for further variable correlation. Non-correlated variables were subsequently tested for seasonality effects using one-way ANOVA and then used in a Bray-Curtis distance-based Redundancy Analysis (db-RDA), which quantifies the impact of the explanatory variables on the microbiome (dis)similarities (Legendre & Anderson, 1999). zOTU relative abundance, environmental and physiological metadata were used for db-RDA using the phyloseq package (McMurdie & Holmes, 2013). The analysis tests the statistical relationship between microbial community composition and the environmental/physiological variables for each coral compartment and host species combination. A model selection tool (ordiR2step() function in the vegan package, *sensu* Blanchet, Legendre & Borcard, 2008) was performed to select the best db-RDA model (i.e., the best explanatory variables) for variation in microbiome composition of each coral compartment (mucus and tissue) in each host species (Johnson & Omland, 2004). The significance of each explanatory variable was confirmed with an ANOVA-like permutational test (function "permutest") for dbRDA. The explanatory value (in %) of significant explanatory variables (e.g. environmental and physiological parameters) on each microbiome was assessed with Variation Partitioning Analysis of the vegan package (Oksanen et al., 2013). A correlation matrix (based on the default Pearson correlation) between the relative abundance of the 20 most abundant microbial families and significant environmental variables was generated using the R package MicrobiomSeq (Ssekagiri, Sloan & Ijaz, 2017), for

239 which significant values were adjusted using the "BH" correction (Benjamini & Hochberg,
240 1995).
241

## 242 Results

### 243 Composition of coral tissue and mucus microbiomes

244 The bacterial 16S rRNA genes of 136 samples, including coral tissue (n = 24 for *A. millepora*; n
245 = 30 for *A. tenuis*), coral mucus layer (n = 24 for *A. millepora*; n = 28 for *A. tenuis*) and seawater
246 (n = 30; used as reference samples) were sequenced and 12,051 zOTUs identified at single
247 nucleotide variants.

248 zOTU richness differed significantly among mucus, tissue and seawater microbiomes
249 (three-way ANOVA; $F_{(2,131)} = 66.93$, $p = 2x10^{-16}$) and between coral species (*A. millepora vs A.*
250 *tenuis*; three-way ANOVA; $F_{(1,131)} = 29.29$, $p = 2.86x10^{-7}$), but not between seasons (three-way
251 ANOVA; $F_{(1,131)} = 3.78$, $p = 0.053$). Seawater harbored the richest microbial community (558
252 zOTU ± 54.6), followed by the mucus (*A. millepora,* 220 zOTU ± 188; *A. tenuis* 511 zOTU ±
253 234) and tissue (*A. millepora,* 125 zOTU ± 31.6; *A. tenuis,* 173 zOTU ± 146; Supplementary
254 Table S1). Alpha diversity based on Shannon Index also differed significantly among
255 microbiomes from different habitats (mucus, tissue and seawater; three-way ANOVA; $F_{(2,131)} =$
256 $45.06$, $p = 1.28x10^{-15}$) and between coral species (three-way ANOVA; $F_{(1,131)} = 17.16$, $p =$
257 $4.63x10^{-5}$), but not between seasons (three-way ANOVA; $F_{(1,131)} = 1.82$, $p = 0.179$). Alpha
258 diversity of mucus samples was not significantly different (Shannon Index: *A. millepora*, 4.18 ±
259 0.83; *A. tenuis*, 5.15 ± 0.69) from seawater samples (Shannon Index: 4.40 ± 0.209;
260 Supplementary Table S1). In contrast, the tissue microbiome was dramatically different to the
261 mucus and seawater microbiomes and harbored the lowest microbial diversity (Shannon Index:
262 *A. millepora*, 3.35 ± 0.63; *A. tenuis*, 3.54 ± 0.84; Supplementary Table S1).

263 The microbial community of all samples (n = 136, including mucus, tissue and seawater)
264 was dominated by sequences affiliated to the phyla Proteobacteria (average relative abundance ±
265 SD; mucus: 44.1 ± 11.5%; tissue: 62.8 ± 2%; seawater: 39.6 ± 3.1), followed in dominance by
266 Bacteroidetes (mucus: 27.5 ± 13.0%; tissue: 9.6 ± 10.9%; seawater: 12.0 ± 11.4%) and
267 Cyanobacteria (mucus: 14.4 ± 9.0%; tissue: 9.8 ± 11.0%; seawater: 38.5 ± 4.0%). Mucus
268 microbiomes for both *Acropora* species (Figure 1) were characterized mostly by members of the
269 family Flavobacteriaceae (average relative abundance ± SD; for *A. tenuis*: 17.3 ± 9.1%; *A.*

*millepora*: 17.3 ± 12.7%), Synechococcaceae (*A. tenuis*: 12.3 ± 7.8%; *A. millepora*: 13.1 ± 10.2%) and Rhodobacteraceae (*A. tenuis*: 5.7 ± 3.0%; *A. millepora*: 6.4 ± 6.4%; Figure 1). In contrast, the tissue microbiome was dominated by the Endozoicimonaceae family (*A. tenuis*: 43.2 ± 31.7%; *A. millepora*: 20.5 ± 19.7%) with additional representation of Flavobacteriaceae (*A. tenuis*: 7.9 ± 9.6%; *A. millepora*: 7.2 ± 9.6%), Synechococcaceae (*A. tenuis*: 5.5 ± 6.8%; *A. millepora*: 12.3 ± 14.5%) and Rhodobacteraceae (*A. tenuis*: 6.5 ± 10.4%; *A. millepora*: 5.3 ± 8.5%; Figure 1) families. Seawater samples were mostly characterized by members of Synechococcaceae (36.6 ± 3.9%) and Pelagibacteraceae (18.6 ± 4.9%), but also by Rhodobacteraceae (8.6 ± 4.8%) and Flavobacteriaceae (8.0 ± 2.6%; Figure 1). Tissue and mucus microbiomes exclusively shared 1,193 zOTU (9.9%), mucus and seawater microbiomes exclusively shared 1,458 zOTU (12.1%), whereas the tissue and seawater microbiome shared only 66 zOTU (0.6%; Figure 2).

Microbial community composition significantly differed among mucus, tissue and seawater (Figure 3; PERMANOVA, $pseudo\text{-}F_{(2,126)} = 14.53$, $p = 0.001$), between *Acropora* species (PERMANOVA, $pseudo\text{-}F_{(1,126)} = 4.42$, $p = 0.001$) and between seasons (PERMANOVA, $pseudo\text{-}F_{(1,126)} = 1.90$, $p = 0.011$). Interaction between species and compartment was also significant (PERMANOVA, $pseudo\text{-}F_{(1,126)} = 3.07$, $p = 0.002$; other interactions were not significant; Supplementary Table S2).

**Factors influencing coral tissue and mucus microbiomes**

Physiological parameters of the tissue (i.e., chlorophyll *a* normalized to protein content, chlorophyll *a* normalized to Symbiodiniaceae numbers, Symbiodiniaceae density normalized to protein content) were not significantly different between host species. Season only significantly affected chlorophyll *a* normalized to Symbiodiniaceae numbers (two-way ANOVA, $F_{(1,78)} = 7.328$, $p = 0.008$).

Out of a total of 20 environmental variables measured for seawater and sediment, 6 variables were non-mutually collinear and were thus included in the db-RDA analysis. Selected variables were salinity, concentration of particulate organic carbon (POC), total suspended solids (TSS), chlorophyll *a* (Chl*a*), ammonium ($NH_4^+$) and the sum of nitrite and nitrate concentrations (i.e., $NO_2^-/NO_3^-$; Supplementary Table S3). None of the variables changed significantly between seasons ($p > 0.05$ for all variables; Supplementary Table S4).

Environmental/physiological parameters investigated in this study explained a limited amount of variation in the microbial community of mucus and tissue of the two *Acropora* species studied (Figure 4). For example, environmental parameters explained 14% (Chl*a*, $NH_4^+$ and $NO_2^-$ /$NO_3^-$) and 10% (POC and $NO_2^-$/$NO_3^-$ ) of the compositional variability for the mucus microbiome in *A. tenuis* and *A. millepora,* respectively (ANOVA-like permutational test for dbRDA; Supplementary Table S5); $NO_2^-$/$NO_3^-$ was the only explanatory environmental variable common to the mucus microbiome of both *Acropora* species (5% of compositional variability explained in each species). In comparison, for the seawater microbiome, environmental parameters ($NO_2^-$/$NO_3^-$, TSS, POC, Salinity and Chl*a*) explained 32% of the compositional variability of the microbiome (Supplementary Figure S2), suggesting greater environmental sensitivity by the microbial community in the seawater compared to the coral-associated communities.

In contrast, tissue microbiomes of *A. millepora* and *A. tenuis* differed substantially in their response to environmental and/or to physiological parameters. While host physiology (i.e., Symbiodiniaceae density normalized to protein contents) and environment (TSS and Chl*a*) explained 6% and 10%, respectively, of the variation of the tissue microbiome in *A. tenuis*, in *A. millepora*, the compositional variation was solely explained (10%) by environmental parameters $NO_2^-$/$NO_3^-$ and TSS; Variation Partitioning Analysis and ANOVA-like permutational test for dbRDA; Supplementary Table S5). TSS was the only explanatory environmental variable common to the tissue microbiomes of both *Acropora* species (total of 5% and 4% in *A. tenuis* and in *A. millepora*, respectively).

**Correlation between bacterial families and environmental/physiological parameters**

The relative abundance of Synechococcaceae in tissues of both *Acropora* species and the mucus of *A. tenuis* was significantly negatively correlated with TSS (p-values = 0.025-0.039; Figure 5 and Supplementary Tables S6 and S7). In contrast, Synechococcaceae was positively correlated to total $NO_2^-$/$NO_3^-$ in both species (mucus of *A. tenuis*, p-value = 0.002, Supplementary Table S7; and tissue of *A. millepora*, p-value = 0.024, Supplementary Table S6). For *A. tenuis*, Synechococcaceae abundance in the tissue correlated negatively with the only significant physiological parameter (Symbiodiniaceae density normalized to protein contents; p-value = 0.025). In the mucus of *A. millepora,* the abundance of Pirellulaceae was positively correlated

with $NO_2^-/NO_3^-$ (p-value = 0.035) and negatively correlated with TSS (p-value = 0.019), while OCS155 was positively correlated to $NO_2^-/NO_3^-$ (p-value = 0.015). Proteobacteria from the mucus of *A. tenuis*, Pelagibacteraceae and Halomonadaceae, were both strongly negative correlated with chlorophyll *a* in the environment (Pelagibacteraceae, p-value = 0.013; Halomonadaceae, p-value = 0.008). Additionally, Halomonadaceae correlated negatively with $NH_4^+$ (p-value = 0.005; Figure 5 and Supplementary Tables S6 and S7).

Tissue-associated Endozoicimonaceae showed a strong significant positive correlation with Symbiodiniaceae density normalized to protein content in *A. tenuis* (p-value = 0.0003). In contrast, in the tissue of *A. millepora*, Endozoicimonaceae were negatively correlated with $NO_2^-/NO_3^-$ (p-value = 0.02), whereas the abundance of Cryomorphaceae family was negatively correlated with TSS (p-value = 0.02; Figure 5, Supplementary Table S6).

## Discussion

Microbial communities associated with corals are continually exposed to fluctuations in the surrounding environment and the physiology of their host. Previous studies have demonstrated changes in the coral microbiome in response to thermal stress (Ainsworth & Hoegh-Guldberg, 2009; Grottoli et al., 2018; Lee et al., 2015; Thurber et al., 2009), ocean acidification (Grottoli et al., 2018; Thurber et al., 2009), organic matter enrichment (Garren & Azam, 2012), bleaching events (Bourne et al., 2008) and other environmental and physiological factors (Glasl et al., 2019a; Guppy & Bythell, 2006; Kelly et al., 2014; Li et al., 2015; Pollock et al., 2018). However, the coral microbiome is not homogenous across the animal and understanding the sensitivity of the microorganisms inhabiting each coral compartment is crucial to better understand the symbiotic associations of corals and their microbiomes. This study highlights compositional differences in the bacterial communities associated with coral mucus and coral tissue, as well as with the surrounding seawater, findings that are largely consistent with previous studies (Apprill, Weber & Santoro, 2016); Bourne & Munn, 2005; Engelen et al., 2018; Pollock et al., 2018; Sweet, Croquer & Bythell, 2011). Furthermore, the high similarity between mucus and seawater microbiomes (i.e., in alpha and beta-diversity, see Supplementary Tables S1 and S2, Figures 2 and 3) and the high dissimilarity between tissue and seawater microbiomes suggests that the mucus microbial community is more strongly influenced by the external environment than the tissue community. Similar results have been reported for other coral species (*Orbicella faveolata*,

*Diploria strigosa*, *Montastraea cavernosa*, *Porites porites* and *Porites astreoides*), where mucus and seawater shared significantly more microbial taxa than those shared by tissue and seawater microbiomes (Apprill, Weber & Santoro, 2016).

Despite the host species-specificity of the coral microbiomes, some bacterial taxa were ubiquitously associated with a particular coral compartment. For example, the mucus of both species was dominated by Flavobacteriaceae and Synechococcaceae, while Endozoicimonaceae dominated the tissue microbiome of all corals. However, overall microbiome composition also showed some overlap between host compartments, consistent with previous reports of overlap between the mucus and tissue microbiomes of other coral species (Engelen et al., 2018; Sweet, Croquer & Bythell, 2011). This intersection is a natural feature of the coral holobiont as both compartments are within the same host and because the constituents of the surface mucus layer are originally produced inside the tissue (Bythell & Wild, 2011). The sharing of some microbial taxa between compartments may also arise due to methodological challenges associated with retrieving samples that are exclusively mucus or coral tissue (Sweet, Croquer & Bythell, 2011). Similar methodological limitations could also obscure differences between the mucus and seawater microbiomes (Brown & Bythell, 2005).

## Drivers of mucus microbiome variation

We hypothesized that the coral mucus microbiome which is in direct contact with seawater would be primarily influenced by environmental parameters and that the tissue microbiome would be most affected by the physiological state of the coral host. Mucus is highly hydrated: mucocyte cells release their secretions in a condensed form which then undergo a massive swelling upon hydration, forming a visco-elastic gel (Brown & Bythell, 2005). Surface mucus can therefore be influenced by the presence of nutrients dissolved in the surrounding seawater (Tanaka, Ogawa & Miyajima, 2010). As expected, environmental factors were influential in shaping the mucus microbiome of both species (*A. millepora* and *A. tenuis*), consistent with recent studies relating changes in the mucus microbiome with environmental perturbations (Li et al., 2015; Pollock et al., 2018). However, the extent of influence from environmental parameters (10% of variation) on the mucus microbiome was much lower than the influence of environment on the seawater microbiome (32% of variation), suggesting that other factors also play a role in modulating the mucus microbiome. For instance, the surrounding environment may interact with

host physiology and together they alter the bacterial community structure of the mucus. Mucus is a nutrient-rich medium fueled by the photosynthetic activity of the Symbiodiniaceae (Brown & Bythell, 2005) and therefore it is expected that some degree of variation in its chemical composition is explained by host-Symbiodiniaceae factors. For example, *A. millepora* and *A. tenuis* at the sampling site (Geoffrey Bay at Magnetic Island) have previously been shown to be associated with distinct Symbiodiniaceae (LaJeunesse et al., 2018; Ulstrup & van Oppen, 2003; van Oppen et al., 2001). *A. millepora* colonies were associated with Symbiodiniaceae in the genus *Durusdinium* (van Oppen et al., 2001) whereas *A. tenuis* was associated to genus *Cladocopium* (Ulstrup & van Oppen, 2003). These correspond to former Symbiodiniaceae clades D and C, respectively. Links between mucus chemical composition and microbiome community structure have been proposed (Tremblay et al., 2011). Physiological factors regulating the dynamics of production and release of the surface mucus layer could also contribute to regulating mucus microbial composition (Glasl, Herndl & Frade, 2016).

Fluctuations of $NH_4^+$, $NO_2^-/NO_3^-$, Chl*a* and POC in the surrounding seawater significantly structured the mucus microbiome in *Acropora* species. Li et al. (2015) and Chen et al. (2011) suggested that rainfall had a crucial effect on bacterial community variation in the coral microbiome, being mostly associated with an increase in the relative abundance of the *Bacilli* group (Chen et al., 2011; Li et al., 2015). In the present study, $NO_2^-/NO_3^-$ (and its collinear variables daylight, particulate nitrogen and grainsize of sediments; Supplementary Table S3) had the greatest influence on microbiome structure, being a significant factor for both studied species, in what is potentially a seasonal pattern. The link between rainfall and increasing nutrients (such as $NO_2^-/NO_3^-$) is well established for inshore reefs (Fabricius, 2005). In the current study, higher amounts of particulate and dissolved nutrients (but a decrease in TSS), were concomitant with an increase in mucus-associated Synechococcaceae, Pirellulaceae, OCS155 and Rhodobacteraceae and a decrease in Halomonadaceae. For instance, Synechococcaceae in the mucus was highly positively correlated with $NO_2^-/NO_3^-$ and negatively correlated with TSS. These findings corroborate previous work in which the abundance of free-living *Synechococcus* in shallow coastal waters decreased significantly under lower nutrient (especially nitrate) and higher TSS concentrations (Uysal & Köksalan, 2006).

Dissolved nutrients, such as nitrogen and phosphorus, can affect coral physiology and drive changes in the associated microbial community (Shaver et al., 2017; Thompson et al.,

2015). For example, organic-rich nutrients from terrestrial run-off negatively affect the health of corals and promote rapid growth of opportunistic heterotrophic bacteria (e.g. Vibrionales, Flavobacteriales and Rhodobacterales), thus affecting the overall composition of the coral microbiome (McDevitt-Irwin et al., 2017; Weber et al., 2012). In our study, abundances of Flavobacteriaceae and Rhodobacteraceae in the mucus of *A. tenuis* correlated with TSS and $NH_4^+$, respectively. The coral holobiont, including cyanobacteria related to *Synechococcus* spp. (Lesser et al., 2004), can also efficiently take up inorganic nitrogen, for example, as nitrogen is required by the photosynthesis production of its Symbiodiniaceae symbionts (Yellowlees, Rees & Leggat, 2008). In fact, $NH_4^+$ can be assimilated by both coral and its Symbiodiniaceae (Pernice et al., 2012), and recent work has implicated bacteria such as *Vibrio* and *Alteromonas* in the incorporation and translocation of $NH_4^+$ into coral tissues and associated Symbiodiniaceae (Ceh et al., 2013). Nitrifying members of the mucus microbiome, such as ammonium oxidizing bacteria (e.g., Pirelullaceae) and archaea, are fueled by $NH_4^+$ (Beman et al., 2007; Siboni et al., 2008; Yang et al., 2013), and $NO_2^-$/$NO_3^-$ can be respired by nitrate reducers putatively active in coral microbiomes (Siboni et al., 2008; Yang et al., 2013). Interestingly, Pirellulaceae abundances in the mucus of *A. millepora* positively correlated with concentrations of environmental $NO_2^-$/$NO_3^-$, the products of ammonium oxidation. These nitrogen-cycling processes mediated by microbes are highly dependent on oxygen availability, but because oxygen concentration in the mucus shows strong diel fluctuations (Shashar, Cohen & Loya, 1993), it is possible that both aerobic (e.g., nitrification) and anaerobic (e.g., denitrification) processes happen within the mucus layer at different times of the day. Temporal dynamics in the coral mucus microbiome are thus likely influenced by the individual and collective metabolic capabilities of the diverse assemblage of microbes and by nutrient availability in the surrounding waters.

## Drivers of tissue microbiome variation

The response of the coral tissue microbiome to environmental and physiological parameters differed between coral species. Whereas the tissue microbiome of *A. tenuis* responded to both environment and host physiology, *A. millepora* responded only to environmental parameters. This difference can be associated to specific features of each species, through which *A. millepora* could modulate the internal environment and create more stable intra-tissue conditions than *A.*

*tenuis* (e.g., via skeletal light modulation, host morphology and tissue thickness, *sensu* Enriquez, Mendez & Iglesias-Prieto, 2005). A non-mutually exclusive alternative explanation is the influence of the algal symbiont (Symbiodiniaceae) genotype associated to the host. Little et al. (2004) investigated Symbiodiniaceae communities associated with *A. millepora* and *A. tenuis* on Magnetic Island demonstrating that the coral-algal endosymbiotic relationship in *Acropora* spp. is distinct between species, dynamic and flexible (corals associate with different Symbiodiniaceae types at different life stages, for example), and contributes significantly to physiological attributes of the coral holobiont. Environmental factors such as seawater temperature can also lead to temporal changes in the symbiont community (Cooper et al., 2011; Howells et al., 2012; Rocker, Willis & Bay, 2012). As the microbiome is strongly associated to the coral holobiont, any disturbance in the host-Symbiodiniaceae relationship may have indirect effects on the microbial composition and its response to environmental and physiological factors. Other studies demonstrate the influence of Symbiodiniaceae on the host microbial community and also support the idea that these two components of the coral holobiont are finely tuned (Glasl et al., 2017; Grottoli et al., 2018; Littman, Bourne & Willis, 2010; Littman, Willis & Bourne, 2009b; Quigley et al., 2019). In the present study, Endozoicimonaceae were strongly positively correlated with the Symbiodiniaceae density in the tissue of *A. tenuis* (see Figure 5). Besides the diversity of Symbiodiniaceae associated to each coral species, other factors can affect the coral and its response to environmental parameters, such as photochemical efficiency (Fv/Fm) and symbiont density (Cunning & Baker, 2014; Da-Anoy, Cabaitan & Conaco, 2019). For instance, Da-Anoy, Cabaitan & Conaco (2019) demonstrated greatest reduction of Fv/Fm in *A. tenuis* in response to elevated temperatures compared to *A. millepora* and, these temperature responses of the corals did not directly correlate with their associated Symbiodiniaceae. This suggests that other species-specific physiological factors could modulate the responses of the coral to the environment and, indirectly, influence the tissue-associated microbiome. One such factor is the way coral-associated microbial aggregates (CAMAs) are distributed throughout the tissue, which varies within populations and can vary among coral species (Work & Aeby, 2014; Wada et al., 2019).

TSS was the only environmental parameter that significantly affected the tissue microbiome of both coral species. TSS can impact corals by limiting light availability for photosynthesis and decreasing Symbiodiniaceae densities, which can indirectly affect microbial

communities (Fabricius, 2005; Pollock et al., 2014). The environment of inshore reefs such as those found around Magnetic Island is characterized by high levels of suspended solids. The decrease in TSS is strongly associated with an increase in the abundance of tissue-associated Synechococcaceae and Cryomorphaceae. Cryomorphaceae are typical copiotrophs in the phylum Bacteroidetes and their increase in the tissue of *A. millepora* could relate to declines in coral holobiont health.

**Conclusions**

This study highlights that microbiomes inhabiting different physical microniches within the coral holobiont differ in their response to host and environmental factors. Microbiomes of *Acropora* spp. differed significantly among host compartments (surface mucus layer and tissue) and species (*A. tenuis* and *A. millepora*) and were also influenced by sampling season. Environmental parameters had the greatest influence on the mucus microbiome in both species whereas the tissue microbiomes showed differential responses to environmental/physiological parameters, suggesting host-specific modulation of the tissue microbiome. By investigating temporal variation in environmental and physiological drivers of the coral microbiome across distinct host compartments in closely related species, we have started to disentangle the factors controlling microbiome composition in corals.

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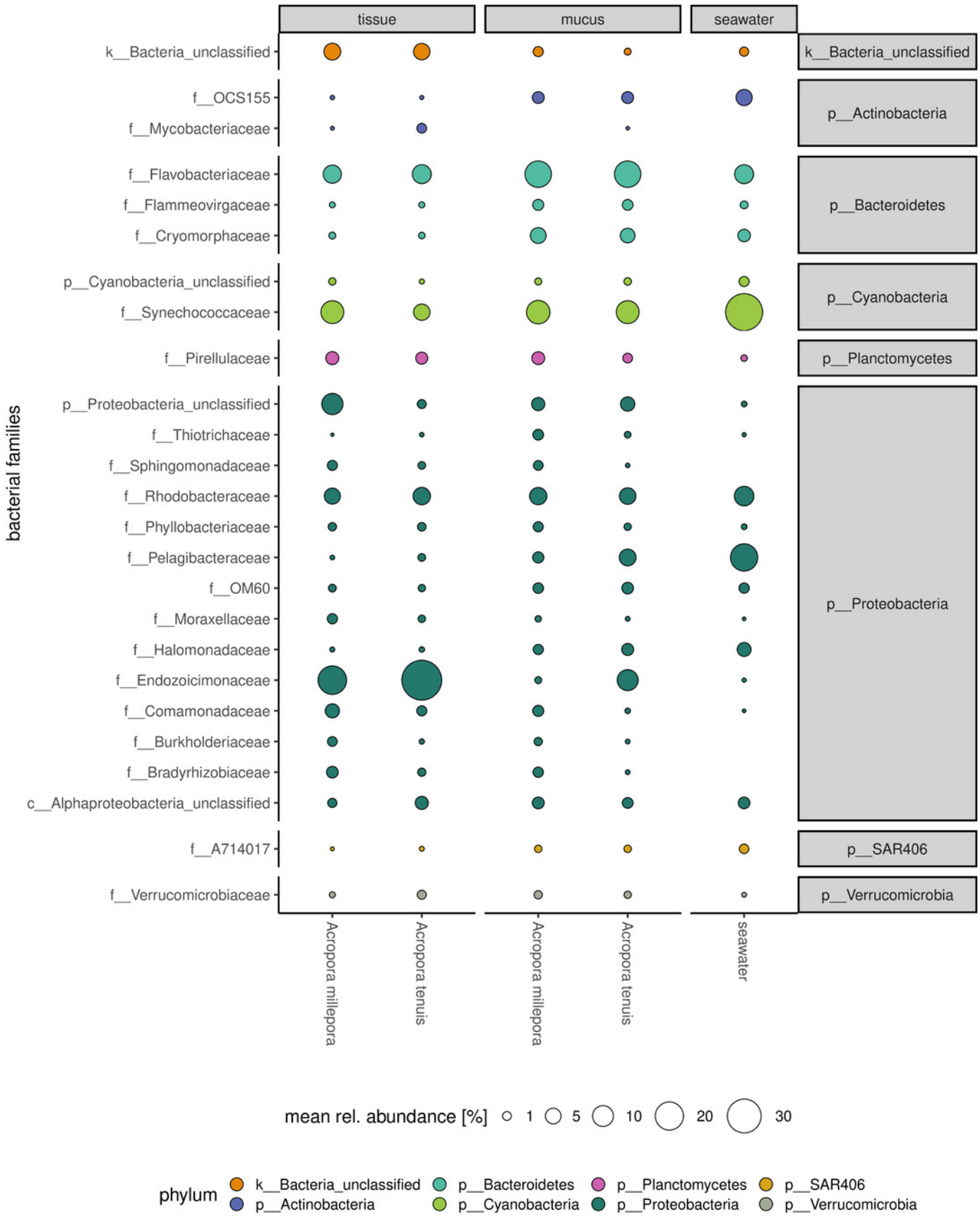
# Figure 1

Compartment-specific microbiome composition of *Acropora tenuis* and *Acropora millepora*.

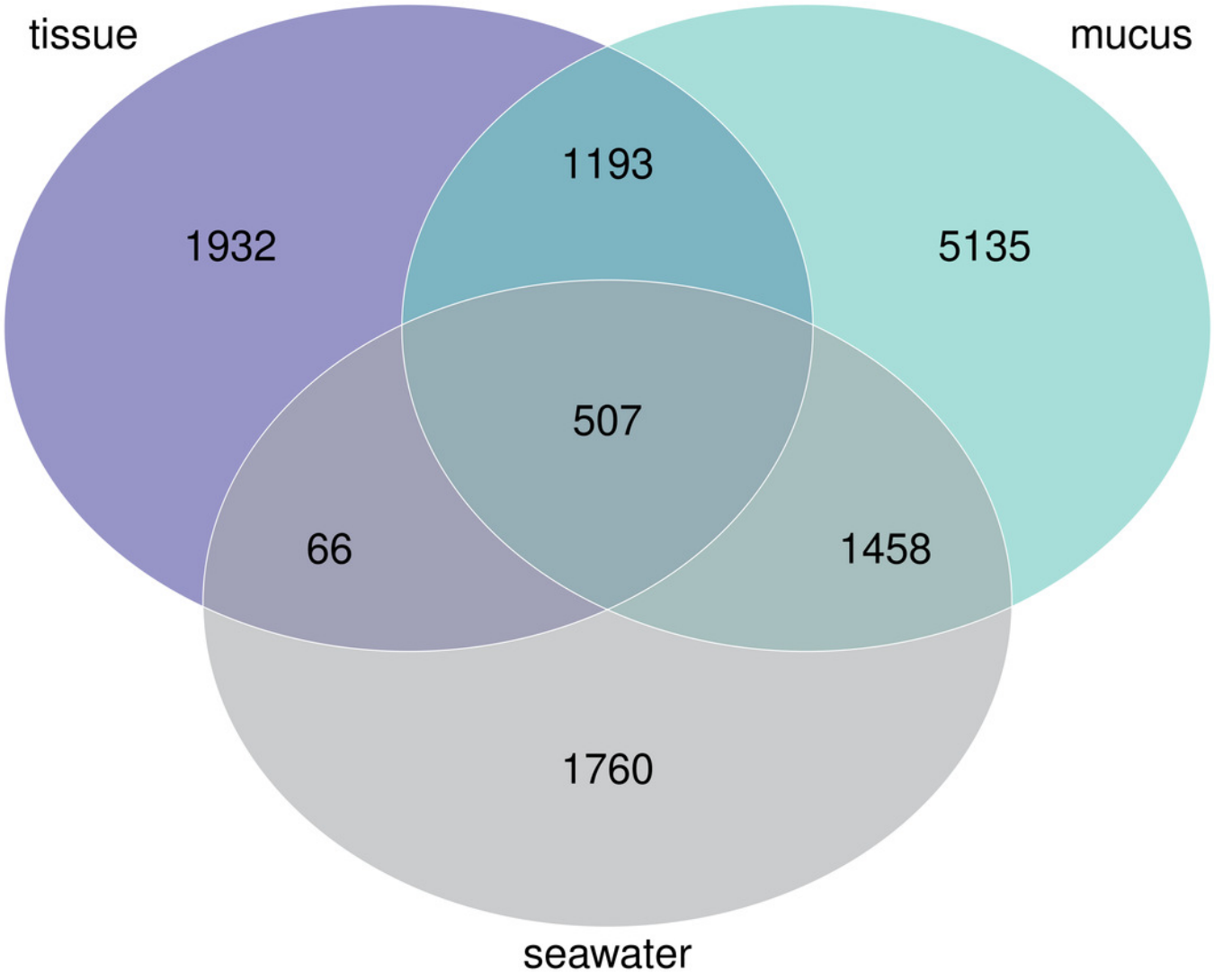
Microbial community composition (mean relative abundance) resolved for the surface mucus layer and tissue of two *Acropora* coral species (*A. tenuis* and *A. millepora*), and surrounding seawater, based on partial 16S rRNA gene amplicon sequencing. Only the 25 most abundant families across all samples are represented, according to their phylum-affiliation (represented by different colors).


tissue
mucus
seawater
k__Bacteria_unclassified
f__OCS155
f__Mycobacteriaceae
f__Flavobacteriaceae
f__Flammeovirgaceae
f__Cryomorphaceae
p__Cyanobacteria_unclassified
f__Synechococcaceae
f__Pirellulaceae
p__Proteobacteria_unclassified
f__Thiotrichaceae
f__Sphingomonadaceae
f__Rhodobacteraceae
f__Phyllobacteriaceae
f__Pelagibacteraceae
f__OM60
f__Moraxellaceae
f__Halomonadaceae
f__Endozoicimonaceae
f__Comamonadaceae
f__Burkholderiaceae
f__Bradyrhizobiaceae
c__Alphaproteobacteria_unclassified
f__A714017
f__Verrucomicrobiaceae
k__Bacteria_unclassified
p__Actinobacteria
p__Bacteroidetes
p__Cyanobacteria
p__Planctomycetes
p__Proteobacteria
p__SAR406
p__Verrucomicrobia
bacterial families
Acropora millepora
Acropora tenuis
Acropora millepora
Acropora tenuis
seawater
mean rel. abundance [%]
1
5
10
20
30
phylum
k__Bacteria_unclassified
p__Actinobacteria
p__Bacteroidetes
p__Cyanobacteria
p__Planctomycetes
p__Proteobacteria
p__SAR406
p__Verrucomicrobia

# Figure 2

Number of shared, unique, and ubiquitous zOTUs among mucus, tissue and seawater microbiomes.

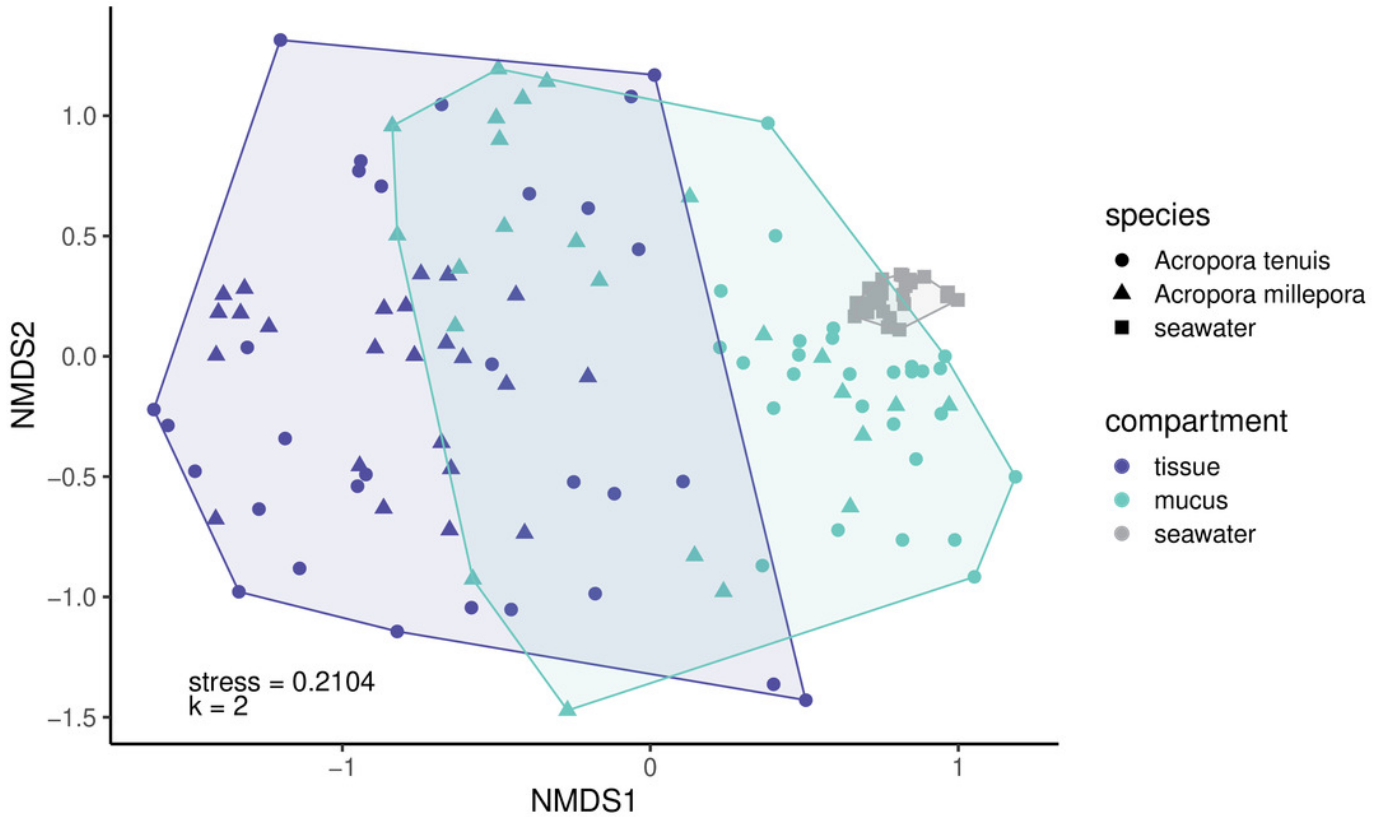
Two *Acropora* species (*A. tenuis* and *A. millepora*) are pooled for the tissue and mucus microbiomes.



# Figure 3

Compositional variability of microbiomes associated with distinct coral-compartments and the ambient seawater.

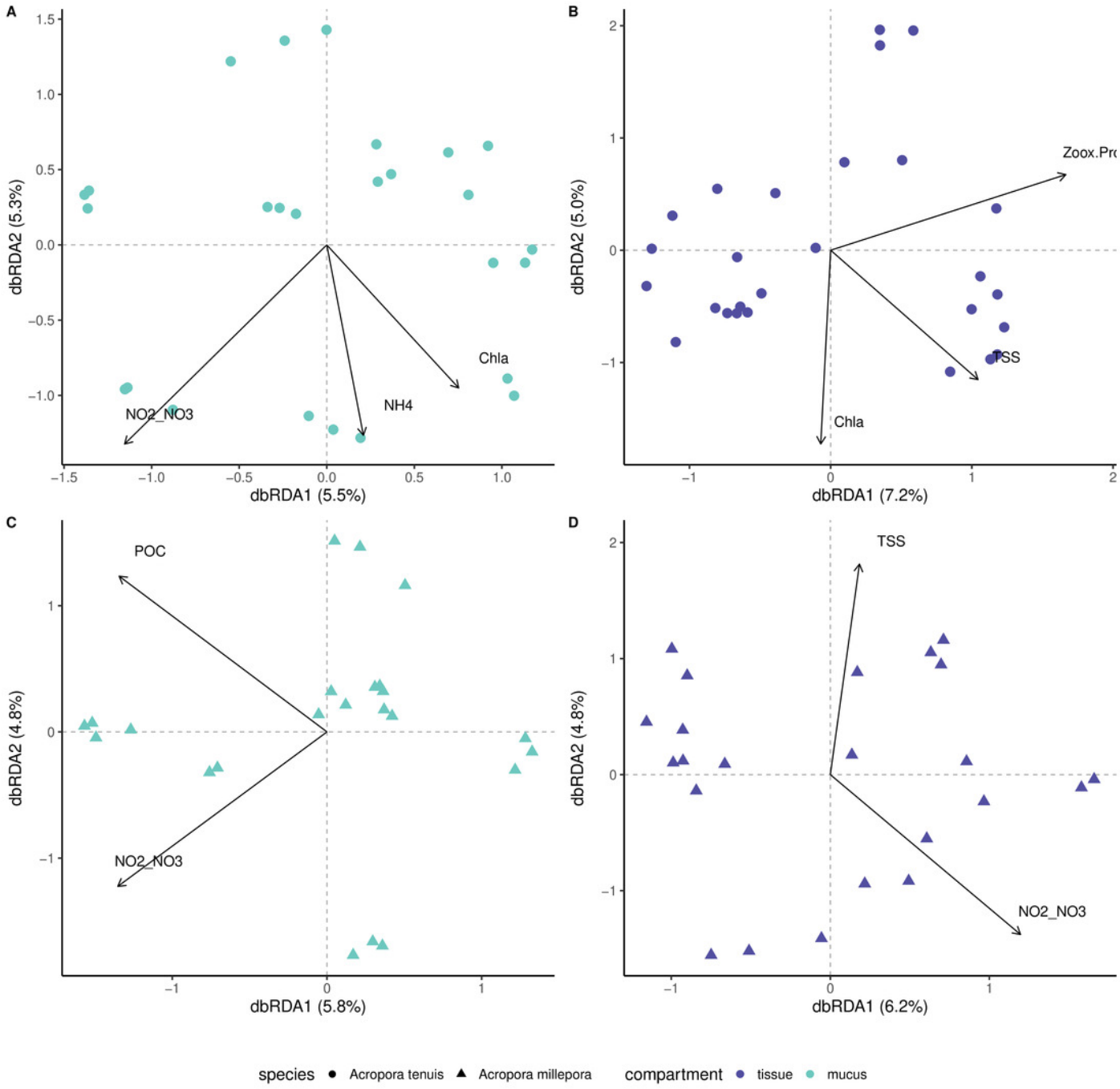
Two-dimensional non-metric multidimensional scaling (nMDS) ordination depicting variation in microbial community structure between coral compartments (mucus and tissue) of *Acropora tenuis and Acropora millepora*, and seawater samples. “k” is the number of dimensions.



# Figure 4

Environmental and physiological drivers of the *Acropora tenuis* and *Acropora millepora* microbiomes.

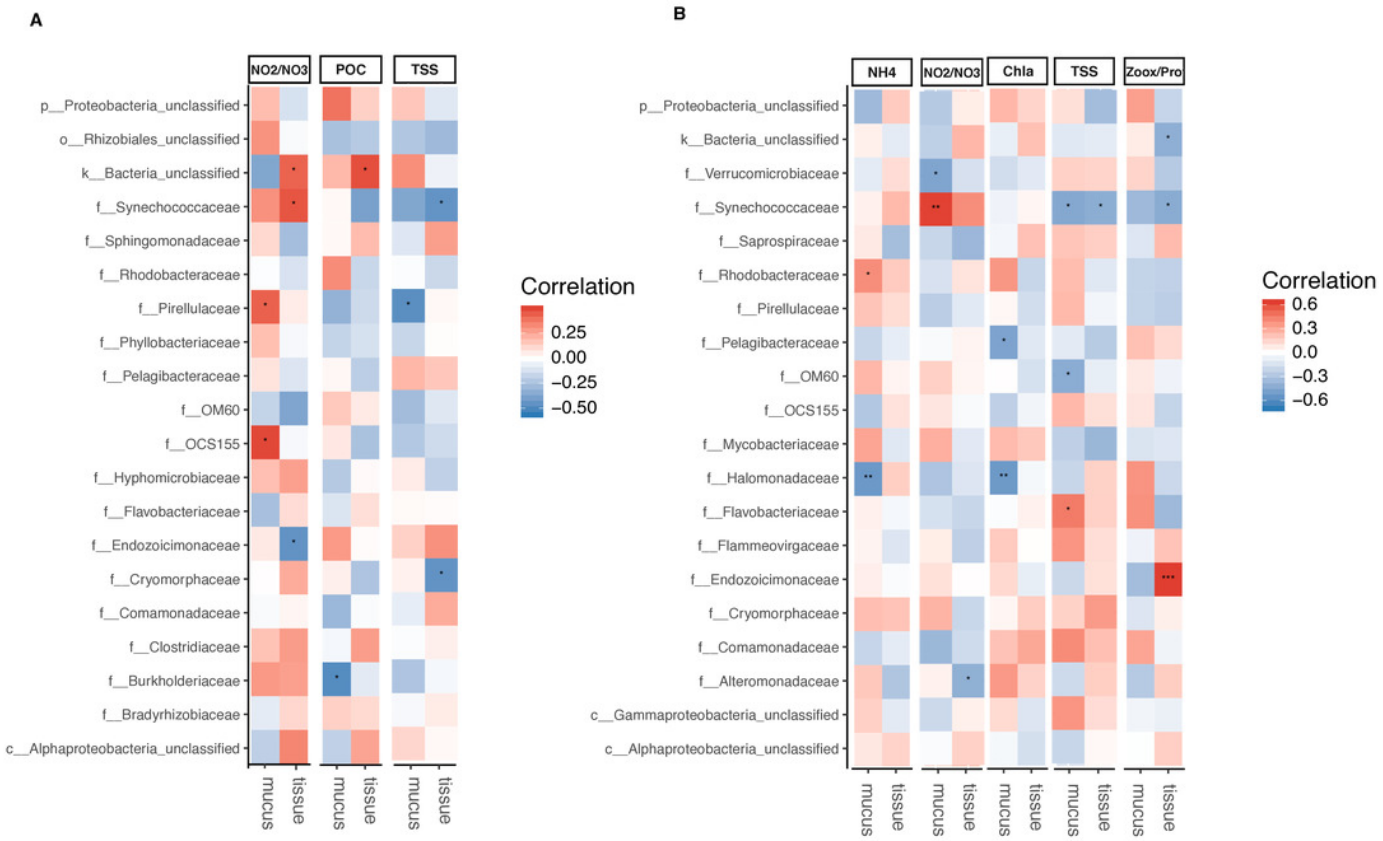
Distance-based redundancy analysis (db-RDA) of the relationship between environmental/physiological variables and the relative abundance of bacteria in (A) mucus and (B) tissue microbiome of *A. millepora*, and (C) mucus and (D) tissue microbiome of *A. tenuis*. Arrow length indicates the strength of the correlation between the variables and the samples (note only significant variables are shown). The selected variables explain a total of (A) 15.0%, (B) 16.4%, (C) 10.6% and (D) 11.0% of the observed variance, respectively. Environmental/physiological variables represented are the sum of nitrite and nitrate concentrations (NO2_NO3), particulate organic carbon (POC), total suspended solids (TSS), ammonium concentration ($NH_4$), chlorophyll *a* concentration (Chla) in seawater and Symbiodiniaceae density normalized to protein contents (Zoox.Pro) of coral tissue.


A
dbRDA2 (5.3%)
dbRDA1 (5.5%)
NO2_NO3
NH4
Chla
B
dbRDA2 (5.0%)
dbRDA1 (7.2%)
Zoox.Pr
TSS
Chla
C
dbRDA2 (4.8%)
dbRDA1 (5.8%)
POC
NO2_NO3
D
dbRDA2 (4.8%)
dbRDA1 (6.2%)
TSS
NO2_NO3
species
Acropora tenuis
Acropora millepora
compartment
tissue
mucus

# Figure 5

Bacterial taxa significantly correlated with environmental and physiological variables.

Pearson's coefficient based correlation matrix between the 20 most abundant bacterial families and environmental/physiological variables having a significant effect on the microbiome associated with tissue and surface mucus of (A) *Acropora millepora* and (B) *Acropora tenuis*. Significant correlations indicated by asterisks at different levels of significance (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$) after correction for multiple comparisons (using BH correction). Environmental/physiological variables represented are the sum of nitrite and nitrate concentrations (NO2/NO3), particulate organic carbon (POC), total suspended solids (TSS), ammonium concentration ($NH_4$), chlorophyll *a* concentration (Chla) in seawater and Symbiodiniaceae density normalized to protein contents (Zoox/Pro) of coral tissue.


A
NO2/NO3
POC
TSS
p__Proteobacteria_unclassified
o__Rhizobiales_unclassified
k__Bacteria_unclassified
f__Synechococcaceae
f__Sphingomonadaceae
f__Rhodobacteraceae
f__Pirellulaceae
f__Phyllobacteriaceae
f__Pelagibacteraceae
f__OM60
f__OCS155
f__Hyphomicrobiaceae
f__Flavobacteriaceae
f__Endozoicimonaceae
f__Cryomorphaceae
f__Comamonadaceae
f__Clostridiaceae
f__Burkholderiaceae
f__Bradyrhizobiaceae
c__Alphaproteobacteria_unclassified
mucus
tissue
Correlation
0.25
0.00
−0.25
−0.50
B
NH4
NO2/NO3
Chla
TSS
Zoox/Pro
p__Proteobacteria_unclassified
k__Bacteria_unclassified
f__Verrucomicrobiaceae
f__Synechococcaceae
f__Saprospiraceae
f__Rhodobacteraceae
f__Pirellulaceae
f__Pelagibacteraceae
f__OM60
f__OCS155
f__Mycobacteriaceae
f__Halomonadaceae
f__Flavobacteriaceae
f__Flammeovirgaceae
f__Endozoicimonaceae
f__Cryomorphaceae
f__Comamonadaceae
f__Alteromonadaceae
c__Gammaproteobacteria_unclassified
c__Alphaproteobacteria_unclassified
mucus
tissue
Correlation
0.6
0.3
0.0
−0.3
−0.6