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Assessing the coral microbiome at the scale of tissue-specific habitats within the coral meta-organism

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As sequencing techniques have advanced and become cheaper in recent years, there has been a rapid increase in the number of studies conducted into the role of the microbiome in coral health, physiology, and response to environmental change. However, there is substantial variation in the methodological approaches applied. For example, DNA extraction protocols and the types of tissues sampled from the coral meta-organism are known to influence the downstream analyses of the amplified microbial communities and subsequently the interpretation of the microbiome diversity, stability and role. Studies have generally focused on whole organisms, in which the coral sampling steps homogenize the meta-organism microhabitats, however other studies targeting specific microhabitats have identified sources of variation specific to distinct compartments of the coral's microbial landscape. Here we present a comparative analysis of methodologies optimized for the generation of coral microbiome data from the coral tissues and whole coral fragments of two commonly studied branching coral genera with distinct tissue structure. We investigate the microbiome of the imperforate *Pocillopora*, where the coral tissue does not penetrate through the calcium carbonate matrix, and the perforate *Acropora*, where the coral tissues and skeleton are interwoven throughout the coral branch. Through comparing data generated from different DNA extraction protocols using fixed coral tissues isolated from the coral skeletal structure with fixed whole coral fragments, we identify sources of variation inherent to microbial data generated from different sample types, species, and extraction protocols.

KEYWORDS

coral microbiome, 16S rRNA gene, amplicon sequencing, DNA extraction, microbial symbiosis, *Pocillopora*, *Acropora*

Introduction

Environmental changes such as rising sea surface temperatures (Hoegh-Guldberg et al., 2007; Hughes et al., 2017), ocean acidification (Hoegh-Guldberg et al., 2007), and pollution (Silbiger et al., 2018) have resulted in substantial degradation to coral reefs worldwide. As reefs worldwide change in both structure and function (McFall-Ngai et al., 2013; Thompson et al., 2015; Ainsworth and Gates 2016; Apprill, 2017; Trevathan-Tackett et al., 2019), it is increasingly important to understand the contribution of the microbiome to the health, physiology, and environmental flexibility of coral species. Most studies investigating the coral microbiome have predominately focused on profiling microbial communities *via* next generation sequencing of 16S rRNA gene amplicons using whole fragments of individual corals, such as sections of branches collected from the colony of a branching coral species. Ultimately, the goal of these studies is to characterize coral-microbe interactions in different coral species (or morphotypes) in response to the combined challenges of environmental change (Stévenne et al., 2021). Considering the unique biotic interactions and dynamic relationships between the microbiome and the environment of morphologically, structurally, and functionally different coral hosts when assessing the response of the coral meta-organism may aid in meeting this goal.

Coral colonies harbor distinct and often complex microbial communities within the niche habitats of the colonial meta-organism (Rohwer et al., 2002; Sweet et al., 2011; Blackall et al., 2015), including the polyp tissue (Mouchka et al., 2010), surface mucus layer (Bythell and Wild, 2011; Glasl et al., 2016), and skeleton (Sweet et al., 2011). Whole coral fragments used for microbial analysis therefore include bacteria not only associated with the coral tissues but also a high number of taxa associated with coral mucus (Shnit-Orland and Kushmaro, 2009; Pereira et al., 2017), the calcium carbonate skeleton (e.g. nitrogen fixing bacteria, Williams et al., 1987; Shashar et al., 1994), and endolithic microbes (Marcelino and Verbruggen, 2016; Marcelino et al., 2017; Marcelino et al., 2018). The tissue and mucus harbor unique reservoirs of prokaryotes, with Apprill et al., 2016 finding 23-49% of sequences identified to be unique to tissues and 31-56% of sequences to be exclusive to mucus. The calcium carbonate skeleton of the coral also harbors a distinct microbial community with up to 80 taxonomic units associated with photosynthetic algae (usually *Ostreobium* spp: Le Campion-Alsumard et al., 1995; Massé et al., 2018; but see Marcelino and Verbruggen, 2016) and cyanobacteria within the endolithic layer (Ainsworth et al., 2010; Marcelino and Verbruggen, 2016; Marcelino et al., 2017; Marcelino et al., 2018). Interestingly, it has been suggested that in some species of coral the tissue is the microbial niche where the microbiota are responsive to different environmental conditions (*Oculina*

patagonica, Rubio-Portillo et al., 2016; *Astroides calcularis*, Biagi et al., 2020), highlighting the importance of targeting the specific microbial community of the tissue in studies of coral response to anthropogenic stressors.

While there is growing awareness of the distinct microhabitats found in corals, studies often utilize whole fragments taken from the colonial coral structure to profile the coral microbiome (e.g. Pratte et al., 2018; Epstein et al., 2019). This approach results in a homogenization of the niche-specific microbial communities of the surface mucus layers, tissue, and skeleton (Rosenberg et al., 2007; Ainsworth et al., 2010). The inclusion of multiple microhabitats in whole coral fragments means that the microbial community composition of these samples includes the microbial communities of these microhabitats. Homogenization of microhabitats may therefore affect comparisons between studies describing and interpreting the role of the microbial community of the coral, depending on the objective of the study, with different methods of sample collection and processing capturing distinct compartments across the coral's microbial landscape (Ainsworth et al., 2015; Neave et al., 2017; Weiler et al., 2018). The actual process of homogenization may also create variability depending on the method employed, e.g. between machine-programmed bead-beating and the manual variability of crushing samples with a mortar/pestle (Hernandez-Agreda et al., 2018), indicating a need for the standardization of homogenization methodology as well as targeting specific microhabitats for comparisons between species. When targeting coral tissues specifically, the effectiveness of tissue removal can vary amongst species, with tissue removal less successful for perforate coral species where the coral tissue penetrates throughout the coral skeleton (≥ 5 mm) (Edmunds, 1994; Edmunds and Gates, 2002). This can lead to potential differences in the microbial community profiles identified between species and subsequently require the application of distinct methodological approaches for different species.

Importantly, all studies comparing coral DNA extraction protocols have been conducted on homogenized whole fragments of coral, and to date there is little known about the importance of DNA extraction protocols on tissue samples. In the present study, we separated fixed coral tissue from whole coral fragments of both *Pocillopora* and *Acropora*, two morphologically distinct species, to investigate if methodological procedures for processing the microbial habitat (e.g. tissue) result in bias when interpreting the host microbiome. To further explore how different methods used in coral microbiome studies influence the microbial communities found in coral tissues versus homogenized fragments in two species, we also investigated three different sample processing approaches used in the DNA extraction steps on both coral tissue and coral whole fragments. Previous studies investigating methodological differences between DNA extraction protocols

have found amplification protocols can influence the community composition (Santos et al., 2012; Baker and Kellogg, 2014; Weber et al., 2017; Bardenhorst et al., 2021), with some protocols resulting in greater microbial richness and coverage of dominant microbial groups (Weber et al., 2017) and others resulting in differing numbers of consistent amplifications (Baker and Kellogg, 2014). Of these studies, none have compared extraction methodologies on paraformaldehyde preserved tissues. A recent study also found all protocols tested suitable for comparing coral microbiomes (Pratte and Kellogg, 2021). The most common goal within coral microbiome studies is to determine if the composition of the microbiome differs significantly between two or more groups of samples, and these studies demonstrate that it is important to identify and account for sources of variation beyond variables that differ between the study groups. In the current study we aimed to determine how the microhabitat sample type and tissue processing steps, from tissue structures of differing coral genera and morphotypes, influence the resulting coral microbiome dataset. By investigating the influence of sample type/microhabitat, host species/morphotype, and DNA extraction protocols on the amplification of DNA from fixed coral tissues and the generation of microbial datasets, we found the greatest differences between the microbial communities of whole fragments versus tissue biopsies, as well as between the tissue biopsies of different species.

Materials and equipment

Sample collection

Branch fragments (~3 cm) of *Acropora muricata* (n = 18) and *Pocillopora damicornis* (n = 9) were collected on snorkel using needle-nose pliers sterilized between samples with 70% molecular-grade ethanol from the shallow fringing reef (1-2 m depth) on the southern side of Heron Island, Australia in 2020 (23.4423°S, 151.9148°E; Permit G19/41974.1). Samples were immediately placed in 50 mL conical tubes filled with preservative. Fragments (~ 3 cm) of *P. damicornis* (n = 9) were also collected on snorkel using needle-nose pliers sterilized between samples with 70% molecular-grade ethanol from the shallow fringing reef (1-2 m depth) of Lord Howe Island in 2019 (31.5553°S, 159.0821°E; Permit MEAA19/206) and immediately placed in 50 mL conical tubes filled with preservative. In both locations, samples were collected from colonies > 3 m apart and separated by distinct sand patches to reduce the chances that colonies were clonal. Fragments were transported in conical tubes filled with preservative (preparation detailed in the following paragraph) to the adjacent research stations following collection (Figure 1A). Species are hereafter referred to as *Acropora* and *Pocillopora*.

Sample preservation

Sterile 16% paraformaldehyde ampules (Electron Microscopy Sciences, cat # 50980487) were used for the preparation of 4% paraformaldehyde by a 1:3 dilution with phosphate buffered saline (PBS) solution (PBS tablets (Invitrogen, USA) in UltraPure DNA/RNA-Free Distilled Water (ThermoFisher Scientific, USA). Coral fragments were then added to 50 mL conical tubes immediately following collection and covered with the 4% PFA solution. After 14 hours, PFA solution was removed and replaced with the DNA/RNA free PBS buffer solution for storage. Samples were stored at 4°C for ~3 – 6 months until sample processing.

Decalcification and tissue biopsies

The coral tissues were isolated from each individual branch fragment (n = 9 *Acropora*, 9 *Pocillopora*) using a DNA/RNA free solution of 20% Ethylenediaminetetraacetic acid (EDTA) (described by Hernandez-Agreda et al., 2018). When compared to other methods of preservation (e.g. snap freezing, salt-saturated dimethyl sulfoxide), samples preserved in PFA and decalcified using the following method yielded comparable results for both numbers of sequences and of generated OTUs (Hernandez-Agreda et al., 2018). Branches were submersed in EDTA solution and incubated at 4°C on a shaker tray, with a complete solution change every 3-4 days over a four-week period. Once the entire calcium carbonate structure was dissolved a tissue biopsy was collected from each coral sample using a 1.5 mm tissue biopsy punch tool (ProScitech Pty Ktd, QLD, Australia). Tissue biopsies were placed immediately into 1.5 mL centrifuge tubes and frozen at -20°C until DNA extraction (~ 1-2 weeks following decalcification).

Extraction method

DNA was extracted from coral samples using three protocols (Figure 1B):

1. QIAamp DNA FFPE Tissue Kit (cat #56404) (FFPE) – specifically designed for purifying DNA from formalin-fixed tissue sections.
2. QIAamp DNA Micro Kit (cat #56304) (Micro) – designed for purification of genomic and mitochondrial DNA from small sample sizes.
3. RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (cat #AM1975) (RecoverAll) – optimized for extraction of nucleic acid from difficult samples, e.g. formalin-preserved samples that can modify protein-protein/protein-nucleic acid crosslinks.

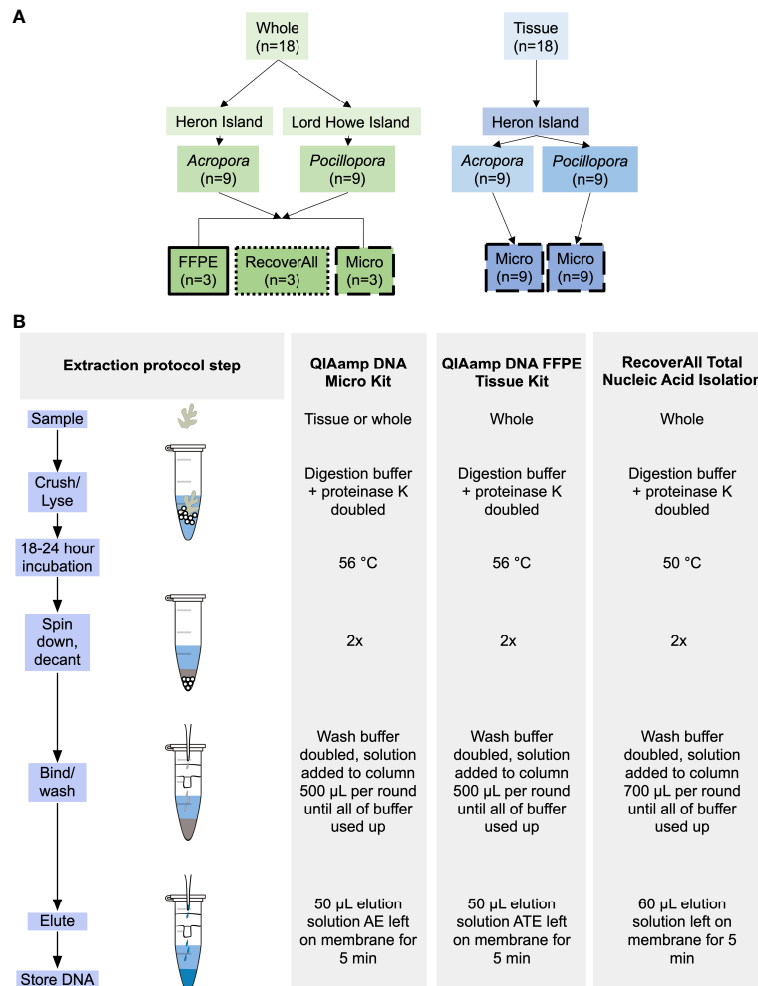


FIGURE 1 Schematics of (A) experimental design: highlighting sample type, species, and extraction protocol, and (B) extraction protocols used in the present study: highlighting subtle differences in methodologies between protocols.

All three protocols were optimized from manufacturer’s protocols to produce the highest quality DNA for sequencing possible (Table S1).

Whole coral fragment sample processing with the FFPE/Micro protocol

Whole fragments of preserved *Acropora* and *Pocillopora* (1-2 cm fragments, n = 3 per species per protocol) were added to 2 mL tubes of 1.4 mm ceramic spheres (Matrix E, MP Biomedicals). Digestion buffers were doubled for all protocols, a step which greatly increased DNA yields following overnight digestion. A FastPrep-24 5G homogenizer (MP Biomedicals, Irvine, CA, USA) was programmed to run 3 rounds of 20 s each (6.0 m/s) to homogenize the sample. The duration and speed of

sampling was chosen because it most effectively homogenized the coral skeleton in the shortest amount of time, e.g. homogenizing the sample fully while minimizing the amount of time the sample was at room temperature. Following homogenization, all samples were incubated overnight (18-24 hours) at 56°C. Samples were then centrifuged (3 min at 10,000 RPM) to pellet calcium carbonate remaining from the coral skeleton. The resulting supernatant was transferred to a new 2 mL tube, centrifuged again to pellet out any remaining calcium carbonate (3 min at 14,000 RPM), and supernatant was transferred to a new 2 mL tube. The centrifugation step was doubled from the manufacturer’s protocol to reduce the chances of calcium carbonate clogging the collection column. AL buffer and 100% molecular-grade ethanol were doubled to increase in proportion to the increased ATL and Proteinase K buffer volume prior to vortexing and adding to the collection column (QIAmp

DNA Mini Kit, Qiagen). Following the manufacturer's protocol for the remainder of the procedure, 500 μ L of buffer solution was added to the collection column and washed until no buffer solution remained and eluted to 50 μ L (Solution ATE for FFPE, Solution AE for Micro).

Whole coral fragment sample processing with RecoverAll protocol

1–2 cm fragments ($n = 3$ per species) were added to 2 mL tubes containing 1.4 mm ceramic spheres (Matrix E, MP Biomedicals). 400 μ L of digestion buffer and 8 μ L of Proteinase K were added prior to bead-beating as described previously. Following homogenization, all samples were incubated overnight (18–24 hours) at 50°C and centrifuged twice (3 min at 10,000 RPM, 3 min at 14,000 RPM) to pellet and remove calcium carbonate remaining from the coral skeleton. Isolation additive and 100% molecular-grade ethanol were doubled to increase in proportion to the increased digestion buffer and Proteinase K buffer volume prior to vortexing and adding to the collection column. The manufacturer's protocol was followed for the remainder of the procedure and DNA eluted to 60 μ L.

Tissue sample processing

1 cm diameter tissue biopsies (1.5 mm thick, $n = 9$ per species) were lysed following manufacturer's protocol for RecoverAll, FFPE, and Micro protocols using corals collected from Heron Island. No mechanical lysis (e.g. bead-beating) step was required for the biopsies, as there was no calcium carbonate skeleton to break down and chemical lysis fully homogenized the sample. Following homogenization, all samples were incubated overnight for 20 hours at 56°C. The manufacturer's protocol was followed for the remainder of the procedure. 1 μ g of carrier RNA was dissolved in buffer AE and added to buffer AL to increase yields for the FFPE and Micro protocols. For elution, 30 μ L of elution solution was incubated at room temperature on the membrane for 5 min before centrifuging the sample for 1 min at 10,000 RPM.

Sample quality and efficiency cutoff

Extracted DNA concentration and purity were quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples with a concentration of equal to or greater than 20 ng/ μ L and a ratio of absorbance at 260/280 nm between 1.5 and 2.3 were sequenced. We were unable to achieve yields and 260/280 ratios that met our sequencing cutoff using the FFPE and RecoverAll protocol on

tissue samples, so only the tissue biopsy samples processed using the Micro protocol were sequenced. Samples processed using the FFPE protocol yielded high 260/280 ratios (> 2.3) and poor DNA resolution, indicative of DNA shredding caused by mechanical disruption. Samples processed using the RecoverAll protocol had yields < 10 ng/ μ L. Extracted DNA was stored at -20°C for ~ 1 week prior to PCR amplification and sequencing.

16S rRNA gene amplicon sequencing and analysis

DNA extraction, amplification, and sequencing were performed on all samples as well as on one negative control (no sample template) prepped for each protocol and sample type cross ($n = 36$ samples, 4 negative controls in total). Sequencing was performed by MR DNA (Molecular Research LP, Shallowater, TX, USA) on the Illumina MiSeq platform following manufacturer's guidelines. The 16S rRNA gene V1–V3 regions PCR primers 27F/519R were used in a 30–35 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 95°C for 5 minutes, followed by 30–35 cycles of 95°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 10 minutes was performed. Samples were multiplexed using unique dual indices, pooled together in equal proportions based on molecular weight and DNA concentrations, and purified using calibrated Ampure XP beads. The pooled and purified PCR product was then used to prepare an Illumina DNA library on an Illumina MiSeq following the manufacturer's guidelines and resulting in 2 x 300 bp paired end sequences.

Sequence data were analyzed using Quantitative Insights Into Microbial Ecology version 2022.2 (QIIME2, [Bolyen et al., 2019](#)). After denoising and primer removal using the DADA2 pipeline ([Callahan et al., 2016](#)) with parameters p-trim-left-f 6, p-trunc-len-f 300, p-trim-left-r 6, p-trunc-len-r 210, taxonomy was assigned to amplicon sequence variants (ASVs) in QIIME2 using a naïve Bayes classifier trained on the Greengenes 13_8 database ([McDonald et al., 2012](#)), a reference database commonly used in microbiome analysis ([Knight et al., 2018](#)). ASVs assigned as “chloroplasts”, “mitochondria”, or unassigned (classification absent at a phylum level) were excluded from the final ASV table. Quality control was conducted in R (version 4.1.0) using the package decontam at a threshold of 0.5, which implements a statistical classification procedure that identifies contaminants in sequencing data ([Davis et al., 2018](#)). In brief, decontam at the 0.5 threshold identifies and filters out contaminants that appear more frequently in negative controls than experimental samples ([Davis et al., 2018](#)). Data exploration was conducted in R using the package phyloseq ([McMurdie and Holmes, 2013](#)). The DESeq2 package ([Love et al., 2014](#)) was used to calculate geometric means of ASV counts prior to estimating size factors using the function *estimateSizeFactors*. The

logfold2change value was then compared between protocols and species using the Wald Test, with the differential abundance measurements considered statistically significant at an adjusted p-value of < 0.05 . The coral core microbiome was determined based on microbe presence in 100% of samples at ASV and genus level (van de Water et al., 2016). Indicator species were identified using the package *indicspecies* (De Cáceres and Legendre, 2009).

Statistics

All statistical analyses were performed in R, using the package *vegan* for multivariate statistics and *ggplot2* for data visualization. Four comparisons were conducted: 1). *Pocillopora* whole fragments * protocol, 2). *Acropora* whole fragments * protocol, 3). *Acropora* samples extracted with the Micro protocol * sample type, and 4). Tissue samples extracted with the Micro protocol * species. Geographically distinct samples (e.g. samples from Heron Island and Lord Howe Island) were not directly compared due to previous evidence showing that bacterial community structures are different between regions and reefs (Hernandez-Agreda et al., 2016). For each comparison, a multiple analysis of variance test (MANOVA) was conducted with extraction protocol, sample type, or species as the main effect and DNA concentration, number of reads per sample, and number of taxa per sample as response variables. For community composition analysis, ASV counts were Hellinger transformed to reduce the effects of numerically large values from overly abundant taxa (Legendre and Gallagher, 2001; sensu Ricci et al., 2022). Alpha diversity metrics were analyzed using separate one-way ANOVAs for each treatment using unrarefied data (Shannon, Chao1, and Inverse Simpson). In cases where residuals were not normally distributed (determined *via* Shapiro test), data was log-transformed to meet assumptions of normality. For beta diversity, metrics included weighted UniFrac, unweighted Unifrac, and Bray-Curtis distance matrices on unrarefied data. A permutational multivariate analysis of variance (PERMANOVA, $n = 9999$, *adonis* function in *vegan*) was performed on each metric to test for dissimilarities in microbial community composition between samples. Homogeneity of dispersion around group centroids was assessed for each metric using PERMDISP (*betadisper* function in *vegan*).

Results

In total, 32,616 sequences from 36 samples and 4 negative controls were generated. Quality control and removal of chloroplasts, mitochondria, unassigned ASVs (classification absent

at a phylum level), singletons, and potential contaminants resulted in the retention of 29,940 sequences with a mean of 785 ± 137 reads per sample, ranging from a minimum of 10 reads to a maximum of 4434 reads. Plotting species richness against sequence sample size for data filtered using *decontam* indicate that adequate depth of sequencing was achieved based on sequencing plateaus (Figure S2). Random subsampling of even numbers of sequences per sample has been criticized in studies with small sample sizes and where the number of sequences per sample vary by 2 or more orders of magnitude (McMurdie and Holmes, 2014), so rarefied data was not used in the present study.

A literature search was conducted to determine reported microbiome sequencing data for relevant coral taxa and methodological approaches used in the current study (Table 1). The total reads and average number of reads per sample were lower than what has been observed for *P. damicornis* and other species within its species complex (Schmidt-Roach et al., 2014), but still within the range of sequencing depth used for analysis of tissue and whole fragment samples of *Pocillopora* spp. (e.g. 412 – 22,900, Table 1). Clustering to 99% similarity and removing negative controls yielded 746 distinct amplicon sequence variants (ASVs) for analysis of the microbial communities present after extraction with each protocol or sample type ($n = 36$ samples). 11 ASVs were removed (from 22 of the 36 samples) as contaminants (Table S1; Figure S3), identified as all sequences more prevalent in negative controls than in samples and reducing total number of reads from 32,616 to 29,940. Between 1-6 ASVs identified as contaminants were removed from each sample. Contaminants were all assigned to the class Alphaproteobacteria and contained the families Caulobacteraceae, Hyphomicrobiaceae, Phyllobacteriaceae, Rhodobacteraceae, Rhodospirillaceae, Acetobacteraceae, Deinococcaceae, and Sphingomonadaceae.

Extraction protocol has limited influence on coral microbiome

No significant differences in alpha diversity metrics were found between extraction protocols for either *Pocillopora* or *Acropora* whole fragments (Figures 3A, B; Table 2). Overall ASV evenness (Shannon diversity) did not differ across protocols for *Pocillopora* samples ($F = 1.750$, $p = 0.252$) or *Acropora* samples ($F = 1.124$, $p = 0.395$). ASV richness (Chao1) was also similar between protocols for *Pocillopora* ($F = 0.415$, $p = 0.678$) and *Acropora* samples ($F = 0.273$, $p = 0.770$). Dominance (Inverse Simpson) also did not differ between protocols for either *Pocillopora* ($F = 2.048$, $p = 0.21$) or *Acropora* samples ($F = 0.564$, $p = 0.597$). Beta diversity and dispersion also did not differ between protocols for either *Pocillopora* or *Acropora* samples

TABLE 1 Summary of a literature search conducted to determine reported microbiome datasets for relevant taxa and methodological approaches used in the current study.

Authors	Year	Species	Sample Size	Total Reads	Avg Per Sample	Depth Used For Analysis	Sequencing Technology Used
Bergman et al.	Present	Pocillopora spp.	Coral tissue (excluding skeleton)	6064	674	Unrarefied	Illumina MiSeq
			Fragment (homogenized)	3119	346	Unrarefied	
		Acropora spp.	Coral tissue (excluding skeleton)	3572	397	Unrarefied	
			Fragment (homogenized)	15506	1722	Unrarefied	
Deignan & McDougald	2022	Pocillopora acuta	Coral tissue (excluding skeleton)	Not Reported	Not Reported	8,416	Illumina MiSeq
Morrow et al.	2022	Pocillopora acuta	Coral tissue (excluding skeleton)	586,015	73,251	3,400	Illumina MiSeq
Ricci et al.	2022	Pocillopora damicornis	Coral skeleton	409,779	68,296	10,000	Illumina MiSeq
			Coral tissue (excluding skeleton)	286,895	47,815	10,000	
Qin et al.	2022	Pocillopora spp.	Fragment (homogenized)	1,741,939	45,840	Not Reported	Illumina MiSeq
Zou et al.	2022	Pocillopora damicornis	Coral tissue (excluding skeleton)	112,970	9,414	Not Reported	Illumina MiSeq
			Surface mucus layer	200,495	16,707	Not Reported	
Bergman et al.	2021	Pocillopora damicornis	Fragment (homogenized)	6,537,144	96,134	22,900	Illumina MiSeq
Caughman et al.	2021	Pocillopora damicornis, Porites lutea, Porites cylindrica	Fragment (homogenized)	Not Reported	36,708	3,038	Illumina MiSeq
Haydon et al.	2021	Pocillopora acuta	Coral tissue (excluding skeleton)	3,241,809	28,688	5,250	Sanger
Li et al.	2021	Pocillopora damicornis	Coral tissue (excluding skeleton)	870,582	12,091	Not Reported	Illumina MiSeq
Vilela et al.	2021	Mussismilia harttii	Polyp	Not Reported	Not Reported	2,180	Illumina MiSeq
Damjanovic et al.	2020	Pocillopora acuta	Fragment (homogenized)	2,152,907	25,630	4,225	Sanger
Epstein et al., 2019	2019	Pocillopora verrucosa	Fragment (homogenized)	3,576,201	309	Not Reported	Illumina MiSeq
Rice et al.	2019	Pocillopora meandrina	Coral tissue (excluding skeleton)	1,108,521	15,396	1,000	Illumina MiSeq
Rosado et al.	2019	Pocillopora damicornis	Fragment (homogenized)	2,022,620	49,332	1,716	Illumina MiSeq
Ziegler et al.	2019	Acropora hemprichii	Coral tissue (excluding skeleton)	894,694	16,267	Not Reported	Illumina MiSeq
		Pocillopora verrucosa		1,508,081	26,930	Not Reported	

(Continued)

TABLE 1 Continued

Authors	Year	Species	Sample Size	Total Reads	Avg Per Sample	Depth Used For Analysis	Sequencing Technology Used
			Coral tissue (excluding skeleton)				
Pogoreutz et al., 2018	2018	Pocillopora damicornis	Coral tissue (excluding skeleton)	3,576,201	2,058	Not Reported	Illumina MiSeq
Pollock et al.	2018	Pocillopora damicornis	Surface mucus layer	109,037	13,629	1,000	Illumina MiSeq
			Coral tissue (excluding skeleton)	65,856	7,317	1,000	
			Coral skeleton	98,355	10,928	1,000	
van Oppen et al.	2018	Pocillopora damicornis	Fragment (Homogenized)	1,240,976	7,348	800	Illumina HiSeq
Aprill et al.	2016	Orbicella faveolata, Montastrea cavernosa, Diploria strigosa, Porites astreoides, Porites porites	Coral tissue (excluding skeleton)	13,200,00	Not Reported	10,000	Illumina MiSeq
			Surface mucus layer		Not Reported	10,000	
			Fragment (homogenized)		Not Reported	10,000	
Glasl et al.	2016	Porites astreoides	Surface mucus layer	639,196	5,810	801	Roche 454 Flex
Meistertzheim et al.	2016	Madrepora oculata	Polyp	37,524	Not Reported	446	Roche 454 FLX
		Lophelia pertusa	Polyp	22,664	Not Reported	446	
Ainsworth et al.	2015	Acropora granulosa	2mm tissue biopsy of coral polyps	326,055	9,880	Not Reported	Roche 454 FLX
		Leptoseris spp.	2mm tissue biopsy of coral polyps	403,563	14,412	Not Reported	
		Montipora capitata	2mm tissue biopsy of coral polyps	131,844	8,240	Not Reported	
Li et al.	2014	Porites lutea	Surface mucus layer	37,387	Not Reported	6,359	Roche 454 FLX
			Coral tissue (excluding skeleton)	48,367	Not Reported	6,359	
			Skeleton	31,598	Not Reported	6,359	
Meyer et al.	2014	Porites astreoides	Surface mucus layer (no lesion)	27,567	2,756	1,133	Roche 454 FLX
			Surface mucus layer (lesion site)	23,066	3,295	1,133	
Bayer et al.	2013	Pocillopora damicornis	Coral tissue (excluding skeleton)	Not Reported	Not Reported	412	Roche 454 FLX
		Stylophora pistillata	Coral tissue (excluding skeleton)	131,421	26,284	18,676	

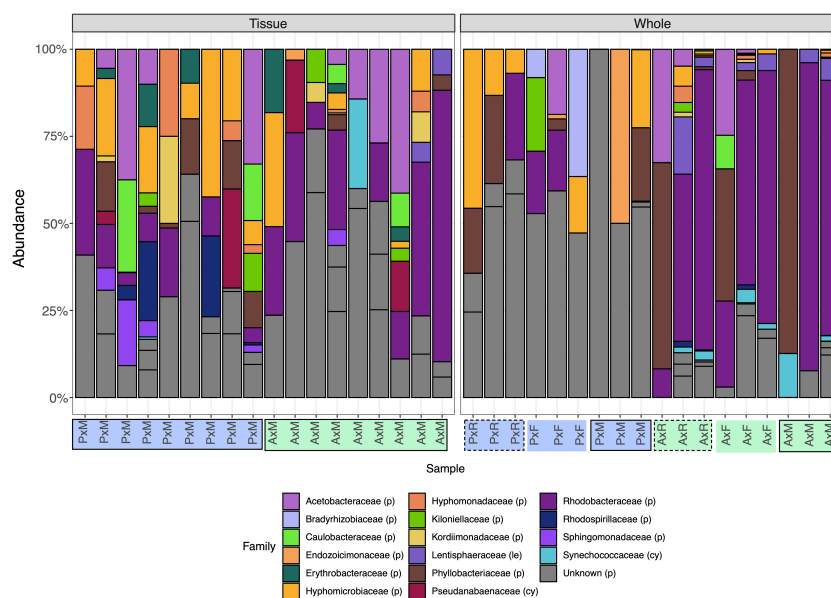


FIGURE 2

Relative abundance of the top 20 bacterial families present in samples from three protocols (M/solid border: Micro, R/dashed border: RecoverAll, F/no border: FFPE), two coral genera (P/blue: Pocillopora, A/green: Acropora), and two sample types (Tissue and Whole). Extractions on tissue biopsies were only successful using the Micro protocol, whereas extractions on whole fragments were successful using the RecoverAll, FFPE, and Micro protocols. Species that had the same taxonomy at the family level were merged using the tax_glom function in phyloseq. Four groups could not be resolved to the family level ("Unknown") and are represented as distinct ASVs by subdivisions of grey columns. Phylum is indicated by either (p): Proteobacteria, (le): Lentisphaerae, or (cy): Cyanobacteria.

using weighted UniFrac, unweighted UniFrac, or Bray-Curtis distance matrices (Figures 4A, B; Table 2). DESeq2 did not identify significant differential abundances across protocols for *Acropora* samples ($p > 0.97$), but one ASV, an unidentified *Devosia* spp., was found to differ across protocols for *Pocillopora* samples ($p < 0.05$). For both *Pocillopora* and *Acropora* whole fragments, there was no difference between protocols for yield, reads, or taxa numbers (Table 3).

Sample type impacts differential abundance in *Acropora* samples

When comparing microbial communities between whole and tissue samples of *Acropora* samples using only the Micro protocol, both whole and tissue samples of *Acropora* were dominated by Alphaproteobacteria (Figure 2), with both the whole fragments and the tissue samples dominated by Rhodobacterales (relative mean abundance of $80 \pm 9\%$ and $28 \pm 6\%$, respectively). No significant differences in alpha diversity metrics were found between whole or tissue sample types (Figure 3C). Chao1 and Inverse Simpson values were log-transformed to meet assumptions of normality. There were no differences between sample types in Shannon ($F = 0.194$, $p = 0.669$), Chao1 ($F = 0.151$, $p = 0.706$), or Inverse Simpson ($F = 0.201$, $p = 0.664$) diversity metrics (Table 2). Dispersion differed

between sample types for Bray-Curtis distance (PERMDISP; $p = 0.007$), but no other beta diversity metrics were significantly different (Figure 4C; Table 2). 4 ASVs were significantly different in whole fragments, all of which were identified to the family Rhodobacteraceae with an average log₂ fold change value of -12.84 ± 0.08 . No difference was found between sample types for yield, reads, or taxa numbers (Table 3). Indicator species could not be identified between sample types, and there were no core taxa shared at 100% prevalence across whole *Acropora* samples or tissue *Acropora* samples using the Micro protocol.

Host species influences diversity and taxa numbers in tissue biopsies

When comparing microbial communities between *Acropora* and *Pocillopora* tissue samples using the Micro protocol, both species were dominated mainly by Alphaproteobacteria (Figure 2). *Pocillopora* tissue samples were dominated by Rhizobiales (relative mean abundance of $35 \pm 8\%$), and *Acropora* tissue samples were dominated by Rhodobacterales (relative mean abundance of $28 \pm 18\%$). Chao1 and Inverse Simpson values were log-transformed to meet assumptions of normality (Table 2). There were no differences between species in Shannon ($F = 2.394$, $p = 0.141$) and Inverse Simpson ($F =$

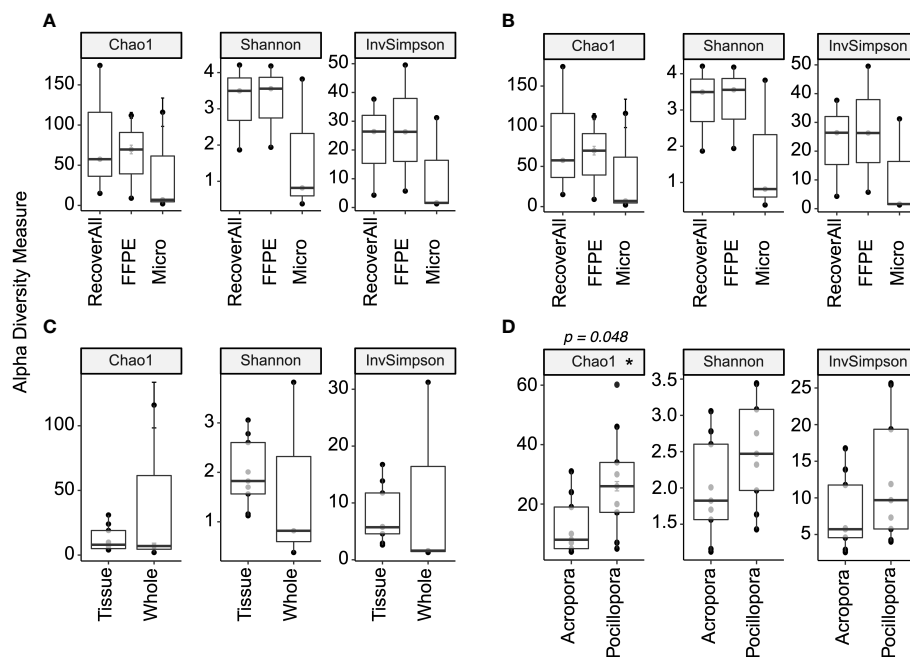


FIGURE 3
Alpha diversity metrics comparing extraction protocols between (A) whole *Pocillopora* samples and (B) whole *Acropora* samples, (C) sample type for *Acropora* using the Micro protocol, and (D) species using tissue samples extracted using the Micro protocol. A star (*) denotes significant differences ($p < 0.05$).

2.068, $p = 0.170$) metrics of diversity, but Chao1 differed between *Acropora* and *Pocillopora* samples (ANOVA; $F = 4.069$, $p = 0.048$, Figure 3D). Beta diversity metrics did not differ between species (Figure 4D; Table 2). Differential abundances were also not significant between species ($p > 0.79$). There was no difference found between species for yields or reads but taxa numbers were significantly higher in *Pocillopora* samples ($p = 0.042$), averaging 29 ± 6 taxa per sample for *Pocillopora* and 13 ± 3 taxa per sample for *Acropora* (Table 4). Indicator species could not be identified between sample types, and there were no core taxa shared at 100% prevalence across *Acropora* or *Pocillopora* tissue samples using the Micro protocol.

Discussion

This study aimed to identify sources of variation in microbial datasets generated from different sample types, species, and extraction protocols by comparing methodologies of coral microbiome data generation. In assessing yields, reads, taxa numbers, and diversity between protocols we find that no single extraction protocol is superior for DNA extractions when using whole coral samples, but only one protocol from the current study is suitable for tissue biopsy samples. We also find significant differences between whole fragments and tissue biopsies of *Acropora*, and between *Acropora* and *Pocillopora*

tissue biopsies, suggesting that method of sample preparation (whole fragment versus tissue biopsy) does influence the coral microbiome based on the biology of the targeted species. We therefore suggest that future studies interpreting the response of the coral microbiome to change target communities of interest, for example by accounting for morphology when selecting species, and therefore conserve the role, function, and significance of the coral-microbe interactions across the coral meta-organism.

All extraction protocols tested met standard DNA quality standards for bacterial 16S amplification from whole fixed *Pocillopora* and *Acropora* fragments, a finding repeated in prior methodology studies (Baker and Kellogg, 2014; Weber et al., 2017; Pratte and Kellogg, 2021). However, in tissue samples from both species, low yields (< 20 ng/ μ L) and high 260/280 ratios (> 2.3) indicative of possible phenol contamination, DNA shredding, or poor sample quality limited extractions to the Micro protocol. It has been suggested that, in samples with low bacterial biomass in relation to overall sample size and DNA content (such as corals), contaminants may be incorrectly identified as novel experimental findings (Glassing et al., 2016; Olomu et al., 2020). Contaminants identified in blank protocol samples were therefore removed from study samples, reducing total number of reads from 32,616 to 29,940 (8% of reads associated with contaminants). Studies that have identified kit contaminants

TABLE 2 Results for all alpha and beta diversity analyses.

Comparison	Diversity Index	ANOVA	PERMDISP	PERMANOVA
Pocillopora (whole) x Protocol	Shannon	p = 0.2520		
	Inverse Simpson	p = 0.210		
	Chao1	p = 0.678		
	Weighted		p = 0.112	p = 0.427
	Unweighted		p = 0.323	p = 0.279
	Bray-Curtis		p = 0.143	p = 0.578
Acropora (whole) x Protocol	Shannon	p = 0.385		
	Chao1	p = 0.770		
	Inverse Simpson	p = 0.597		
	Weighted		p = 0.873	p = 0.743
	Unweighted		p = 0.998	p = 0.843
	Bray-Curtis		p = 0.873	p = 0.910
Acropora (Micro protocol) x Sample Type	Shannon	p = 0.669		
	Chao1	p = 0.706		
	Inverse Simpson	p = 0.664		
	Weighted		p = 0.318	p = 0.196
	Unweighted		p = 0.781	p = 0.191
	Bray-Curtis		p = 0.007	p = 0.111
Tissue Samples (Micro protocol) x Species	Shannon	p = 0.141		
	Chao1	p = 0.048		
	Inverse Simpson	p = 0.170		
	Weighted		p = 0.120	p = 0.058
	Unweighted		p = 0.121	p = 0.122
	Bray-Curtis		p = 0.484	p = 0.426

Results in italics were log-transformed prior to analysis, and results in bold are significant.

TABLE 3 Results of MANOVA comparing number of taxa at ASV level, yield, and number of reads for each combination of coral species, protocol, and sample type tested in the present study.

Comparison	Number of Taxa	Yield (ng/ μ L)	Number of Reads
Pocillopora (whole) x Protocol	p = 0.672	p = 0.125	p = 0.511
Acropora (whole) x Protocol	p = 0.670	p = 0.051	p = 0.8494
Acropora (Micro protocol) x Sample Type	p = 0.228	p = 0.670	p = 0.240
Tissue Samples (Micro protocol) x Species	p = 0.042	p = 0.77	p = 0.183

acknowledge that many of the contaminants detected can also be due to laboratory/cross-contamination or genuinely present in samples (Kellogg, 2019) so we conservatively removed only 11 taxa that were found to be more present in negative controls than in study samples. While read counts were relatively low for all samples, with 22% of samples (8 out of 36) having below 100 reads, these low reads were evenly distributed across protocols, species, and sample types and no clear pattern in read counts emerged. The number of read counts reported in this study includes samples with both large and small amounts of starting

material, e.g. 1 cm diameter tissue biopsies (skeletal material removed prior to biopsy) and 3 cm branch fragments (from which 1 – 2 cm were used for DNA extraction). This is reported similarly to other studies where sample sizes with different amounts of starting material are analyzed, e.g. where surface mucus layer, tissue, and skeleton show significant differences from each other (Sweet et al., 2011). Consistently, tissue samples excluding skeletal material show lower read counts than whole fragments or mucus samples (Table 1, Meistertzheim et al., 2016; Zou et al., 2022). Specifically, for *P. damicornis*, read counts per sample as high as 96,134 counts have been recorded in the literature for whole fragments (Bergman et al., 2021), compared to 9,414 – 12,091 for tissue samples excluding coral skeleton (Li et al., 2021; Zou et al., 2022). Pollock et al. (2018) also found an average of 13,629 reads per sample in the *P. damicornis* mucus layer and 10,928 reads per sample in the *P. damicornis* skeleton, compared to only 7,317 reads per sample in the *P. damicornis* tissue, highlighting how sample size and read count per sample may bias the overall read count. In addition, similar to Clinton et al., 2021 study on fish gills, clear plateaus in the present study were seen in sequencing curves following filtration, suggesting that even where reads were < 1000 an adequate sequencing depth was reached. No significant differences were found

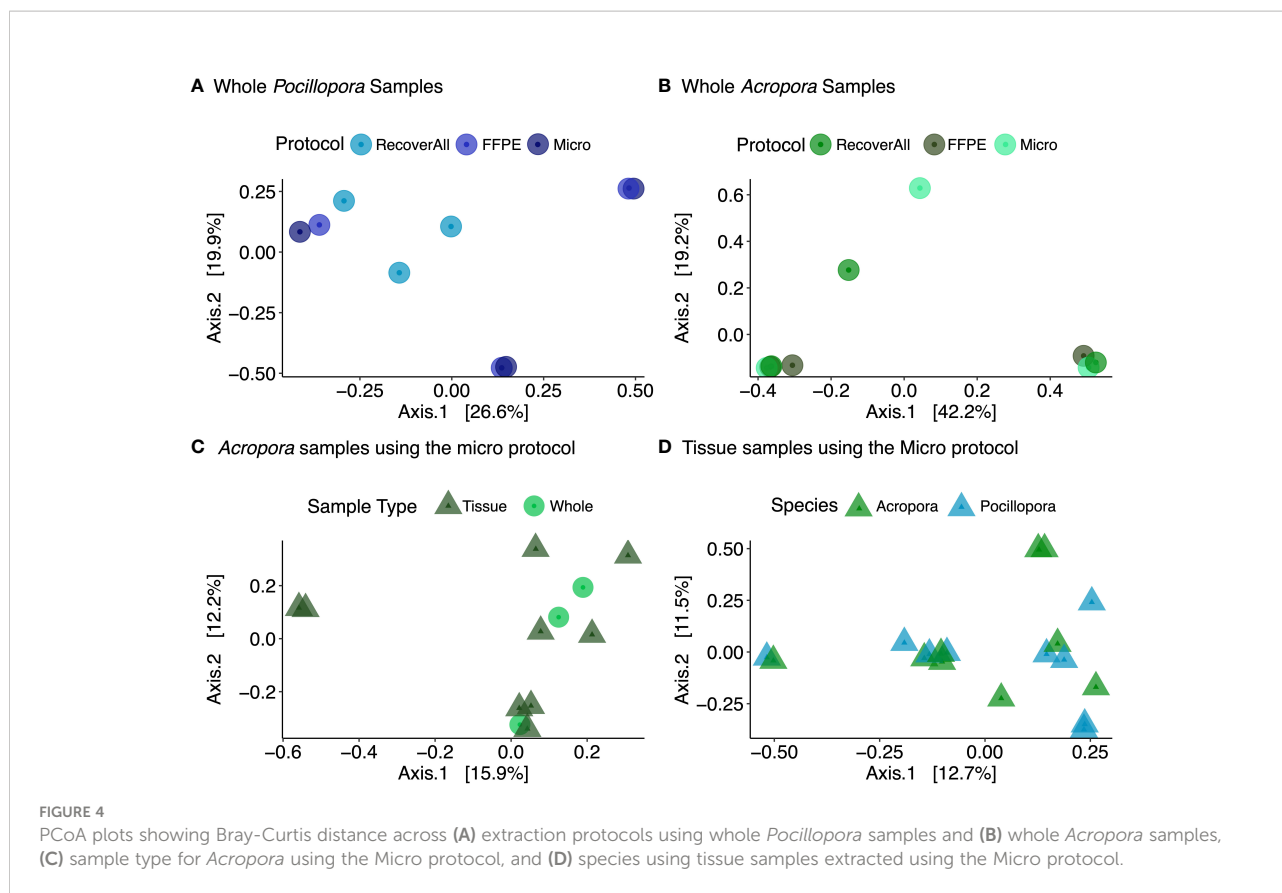


FIGURE 4
PCoA plots showing Bray-Curtis distance across (A) extraction protocols using whole *Pocillopora* samples and (B) whole *Acropora* samples, (C) sample type for *Acropora* using the Micro protocol, and (D) species using tissue samples extracted using the Micro protocol.

between any alpha or beta diversity metrics across protocols for whole fragments, but one taxa differed in relative abundance in *Pocillopora* whole fragments across protocols. The only taxa observed to differ across protocols for *Pocillopora* samples, a *Devosia* spp. found enriched in samples using the Micro protocol, has been identified in the Clade C *Symbiodinium* core microbiome in corals previously (Lawson et al., 2018), suggesting that protocol choice has little bias on the bacterial community of whole *Pocillopora* samples but may be better

suitable to recover sequences of bacteria closely associated with Symbiodiniaceae.

Only the Micro protocol was found to be suitable for use with coral tissue samples. Similarly to Pratte and Kellogg, 2021, all protocols for whole fragments here used bead-beating, which has been found to result in higher DNA concentrations and higher degrees of microbial diversity than other cell lysis methods (Lim et al., 2018; Pollock et al., 2018) and for tissue samples bead beating was excluded. Pratte and Kellogg (2021)

TABLE 4 Table of reads, taxa, and mean yields for each coral species, protocol, and sample type.

Kit	Micro		FFPE		RecoverAll			
	Tissue type		Tissue type		Tissue type			
	Whole Fragment		Tissue		Whole Fragment		Whole Fragment	
Site Species	Heron <i>Acropora</i>	Lord Howe <i>Pocillopora</i>	Heron <i>Acropora</i>	Heron <i>Pocillopora</i>	Heron <i>Acropora</i>	Lord Howe <i>Pocillopora</i>	Heron <i>Acropora</i>	Lord Howe <i>Pocillopora</i>
Min # of reads	103	20	62	107	208	175	255	230
Max # of reads	3491	1147	1336	1697	3254	271	4434	959
Total # of reads	3698	1194	3868	6931	5844	641	6030	1973
Mean # reads/sample ± SE	1243 ± 1139	413 ± 388	429 ± 154	770 ± 188	1974 ± 918	219 ± 28	2096 ± 1292	665 ± 219
# of taxa	100	44	103	218	156	22	235	36
Mean yield ng/μL ± SE	160 ± 51	139 ± 31	144 ± 13	105 ± 16	264 ± 60	197 ± 67	60 ± 4	50 ± 11
# of samples	3	3	9	9	3	3	3	3

also hypothesized that the inclusion of a bead-beating step in all extraction methods contributed to very few significant differences between methods, and we therefore hypothesize that the exclusion of a bead-beating step for the tissue samples (chemical lysis only) could explain the differences in effectiveness between protocols for the tissue samples. However, the addition of a bead-beating step in preliminary trials (Table S2) did not increase yields, suggesting that the chemical lysis steps in the FFPE and RecoverAll protocols were not compatible with mechanical lysis steps for tissue samples in the present experiment. There is substantial evidence in the literature in both coral reefs (Pratte and Kellogg, 2021) and other systems that the effect of extraction protocol on bacterial sequencing result is small, and often nonexistent, when compared to variability across samples, species, and sample types (Wagner Mackenzie et al., 2015; Marotz et al., 2017). By comparing extraction protocols across different sample types, we find here that the effect of sample type (e.g. whole fragment versus tissue biopsy samples) had the greatest influence on extraction protocol success, and therefore more closely examined what influence sample type has on microbial community.

The microbiome of *Acropora* tissue biopsies was also found to differ from that of the whole coral fragment. The tissue and skeletal microbiomes of scleractinian corals have been found to be dominated by rare taxa and to differ in bacterial abundance (Ricci et al., 2022), so we expect to see some differences between samples including the coral skeleton and only including the tissue. Here we find a higher proportion of Rhodobacteraceae in whole fragments than tissue biopsy samples, possibly due to the inclusion of different microhabitats in the two sample types. Rhodobacteraceae have been shown to demonstrate environmental flexibility (Röthig et al., 2016) and have also been associated with thermally-stressed corals, including *Porites* (Pootakham et al., 2019), *Montipora* (van de Water et al., 2017), *Acropora* (Lee et al., 2017), and *Pocillopora* (Tout et al., 2015). However, without separating out the microhabitats from the whole fragment, it is impossible to specify what microhabitat certain bacteria are associated with or draw conclusions about the response of a specific microhabitat to possible environmental change. The advantage of tissue samples therefore is that the microbial community is limited to bacteria found in the coral tissue, not composed of a disproportionate number of bacteria from the SML, coral skeleton, and endoliths that may be included in whole fragment samples, and therefore studies can exclusively target the response of tissue-associated microbes to environmental change.

In the current study, tissue biopsies were standardized to a 1.5 mm wide biopsy punch and we find there were significantly more taxa and a higher diversity of taxa associated with *Pocillopora* tissue samples than *Acropora*. Distinct morphological, reproductive, and ecological traits between species can influence microbial associations in corals (Hernandez-Agreda et al., 2018; Ricci et al.,

2022; Morrow et al., 2022). The species selected in the current study have different morphological structures: *Acropora* species are perforate, characterized by tissue integrated within the calcium carbonate skeletal matrix (Franzisket, 1970; Leuzinger et al., 2003; Muller-Parker et al., 2015), whereas *Pocillopora* are imperforate with tissues located superficially (Muller-Parker et al., 2015). Perforate corals are more likely to have concealed tissue areas shaded from a combination of temperature and light stress (Shashar et al., 1997) and have tissues up to five times thicker than imperforate corals (Yost et al., 2013). The functional traits of coral species (e.g. growth form, disease susceptibility) have also been found to have a stronger influence on tissue and skeletal microbiomes than mucus (Pollock et al., 2018), and we therefore hypothesize that distinct differences between host traits explain the variation between the taxa found in *Pocillopora* and *Acropora* tissue samples in the present study. In the present study, we observed significantly more taxa in *Pocillopora* tissues than in *Acropora* tissues. *Pocillopora* tissue samples were dominated by the family Rhizobiales, nitrogen-cycling bacteria capable of fixing and providing a source of organic nitrogen when in symbiosis with a plant host (Carvalho et al., 2014). On coral reefs, Rhizobiales have been reported to increase on reefs impacted by stressors such as climate change, water pollution, and overfishing (McDevitt-Irwin et al., 2017). As coral samples were collected from a shallow (1-3 m depth) reef flat during summer, one possibility in the present study is that surface-level *Pocillopora* tissue was more impacted by light and temperature (and therefore saw an increase in microbial taxa and diversity, e.g. Tout et al., 2015) than *Acropora* tissue located deeper in the skeleton. Other studies have also amplified Rhizobiales in association with coral tissue, but not in the whole coral colony community (Ainsworth et al., 2015; Morrow et al., 2022), suggesting that characteristics of *P. damicornis* tissue results in higher abundances of Rhizobiales observed here. Additionally, it is possible that preservation method (e.g. fixed tissues) did not preserve communities from the surface mucus layer, which is known to be rich in Acroporid corals and has been found to host opportunistic pathogens and coral-associated commensal bacteria (Krediet et al., 2009; Lee et al., 2015; Hadaidi et al., 2017). For example, Ziegler et al. (2019) identified a mean number of 237 OTUs per sample associated with *Acropora hemprichii* tissues and 159 OTUs per sample associated with *Pocillopora verrucosa* tissues, but the airbrushed tissue slurry from frozen fragments used for 16S rRNA amplicon sequencing included the mucus layer and likely accounted for the higher number of taxa in *Acropora* samples than *Pocillopora*. Liang et al. (2017) also found slightly higher mean taxa numbers associated with *Acropora* spp. tissues (558 OTUs) than *Pocillopora* spp. tissues (523 OTUs) in samples using airbrushed tissue like Ziegler et al. (2019). In the present study, we observed fewer taxa per sample, likely also due to the lower number of reads per sample. For example, in fixed tissue samples, we found an average of 11 ASVs associated with *Acropora* samples and an average of 24 ASVs associated with *Pocillopora* samples. The exclusion of the rich surface mucus layer of *Acropora* corals in

the present study could therefore account for the lower taxa and read numbers observed overall in the present study, as well as the lower number of taxa and taxa diversity observed in *Acropora* corals.

The communities observed in *Acropora* and *Pocillopora* samples were likely also influenced by both preservation method and anthropogenic factors. While Alphaproteobacteria dominated the microbiome of both *Acropora* and *Pocillopora* in the present study, Bergman et al. (2021) previously found Gammaproteobacteria to dominate the microbiome of whole *Pocillopora* fragments. The samples in the present study were collected directly from the reef, whereas Bergman et al. (2021) collected corals and conducted an ex situ tank experiment using multiple warming trajectories, which likely influenced the differences from the microbial communities observed herein due to environmental effects. Additionally, both *Acropora* and *Pocillopora* samples were almost entirely lacking *Endozoicomonas*, a common bacterial associate in corals (Pogoreutz et al., 2018; Epstein et al., 2019; Glasl et al., 2019; Voolstra and Ziegler, 2020) that has been observed to be highly abundant in both *Acropora* and *Pocillopora* species (Ziegler et al., 2019). However, Ricci et al. (2022) characterized the microbiome of *P. damicornis* from Heron Island just a few months before the 2020 bleaching events and found a high abundance of *Endozoicomonas*. The difference between our results and Ricci et al. (2022) suggests that warming water can disrupt the coral-*Endozoicomonas* association and is also supported by Botté et al. (2022) results, who found low abundances of *Endozoicomonas* in *P. acuta* samples from the GBR. As a loss of *Endozoicomonas* is often recorded in bleached or diseased corals (Bayer et al., 2013; Glasl et al., 2016), one possibility for the lack of *Endozoicomonas* recorded herein is that repetitive and severe bleaching on the GBR has greatly reduced populations of coral tissue-associated *Endozoicomonas* over time.

Additionally, the present study investigated the microbiome of corals using fixed tissue, whereas many studies investigating the *Pocillopora* or *Acropora* microbiome analyze whole fragments snap-frozen in liquid nitrogen (e.g. Epstein et al., 2019; Ziegler et al., 2019; Bergman et al., 2021). While Hernandez-Agreda et al. (2018) found composition of microbial communities to be comparable between fixed and snap-frozen coral samples, some low abundance and low occurrence phylotypes were found to be variable between preservation methods. It is therefore possible that variation in the microbial community between studies, as well as number of reads and ASVs, is at least in part due to preservation method. Preservation method is a potential source of bias that should be considered when conducting experiments targeting the coral microbiome. Through investigations of the influence of sample type, host species, and DNA extraction protocols on the amplification of DNA from coral tissues, we find that differences in the microbiome are therefore potentially related

to both the structure and preservation of the tissue of the tissue, skeleton, and distinct environmental niches within the two coral types.

Conclusions

All three protocols used in this study were found to be suitable for the extraction of coral DNA for sequencing from whole fragments and the Micro protocol to be suitable for use on tissue biopsies. However, greater differences were detected between the microbial communities of whole fragments versus tissue biopsies for *Acropora* species, as well as between the tissue biopsies of different species. Similar results have been observed in *Acropora granulosa*, *Leptoseris* spp., and *Montipora capitata*, where different bacterial phylotypes were observed between whole coral samples (e.g. including the surface mucus layer and the coral skeleton) and isolated coral tissues (Ainsworth et al., 2015). Ricci et al. (2022) also found that host phylogeny and microhabitat niches (e.g. tissue slurry samples versus skeletal samples) shaped the presence and relative abundance of bacterial symbionts in 12 coral species. Observation of species-specific and microhabitat-specific bacterial associations therefore highlight the need for studies to incorporate the biological and morphological features of the coral host into interpretations of the coral-microbial community interaction. Similarly, the type of microhabitat targeted can influence how the microbiome is interpreted in studies of the contribution of the microbiome to coral health and physiology as the environment changes. Future studies with a larger sample size than the present study ($n = 3$ coral whole fragments per species/extraction protocol) may find more differences at a community level, however studies of coral response to environmental change are often limited by permitting requirements and therefore often proceed with small sample sizes. To maximize the chances of capturing the microbial community of interest from a small sample size, we therefore suggest that studies targeting the microbial community of coral tissue exclusive of the meta-organism microbial community use tissue biopsies. Additionally, while surveying the literature for reported sequencing depths across *P. damicornis* tissue and whole fragments, we found that bacterial microbiomes often reported as “tissue-associated” were actually referring to homogenized whole fragments. In future studies, tissue biopsies and clear reporting of the community of interest may reduce some of the noise currently existing in coral microbial datasets. Finally, sequencing depths, average number of sequences per sample, and total number of reads for each sample variable category tested were often underreported in the literature (Table 1), pointing to a need for a consistent standard of reporting across coral microbiome studies. As DNA sequencing methods become more and more prevalent in studies of the coral microbiome, it is imperative to identify sources of bias and conduct experiments with targeted communities of interest in order to identify

ecologically meaningful patterns in an often-complicated dataset. Understanding where bias may be introduced throughout different steps in the experimental design, and weighing out the costs and benefits of reducing these biases, will improve our understanding of the coral microbiome and the sequencing process as a whole.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA802894.

Author contributions

JB, TS, and TA planned the experiments. JB and TS conducted the experiments. JB, TS, and TA contributed to the experiments. JB analyzed the data and wrote the manuscript with advice, contributions, review, and editing from TA, SE, and TS.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.985496/full#supplementary-material>

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