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Author/s:
Dungan, AM;Tandon, K;Jameson, V;Gotze, CR;Blackall, LL;van Oppen, MJH

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A targeted approach to enrich host-associated bacteria for metagenomic sequencing

Ashley M. Dungan [1,*], Kshitij Tandon [1], Vanta Jameson[2], Cecilie Ravn Gotze[1,3], Linda L. Blackall[1], Madeleine J. H. van Oppen[1,3]

[1]School of BioSciences, University of Melbourne, Melbourne, VIC 3010, Australia
[2]Melbourne Cytometry Platform, Department of Microbiology and Immunology, University of Melbourne, Melbourne, VIC 3010, Australia
[3]Reef Recovery, Restoration and Adaptation Program, Australian Institute of Marine Science, Townsville, QLD 4810, Australia
*Corresponding author. School of BioSciences, University of Melbourne, Melbourne, VIC 3010, Australia. Tel: +61 403 442 289; E-mail: adungan31@gmail.com
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Abstract

Multicellular eukaryotic organisms are hosts to communities of bacteria that reside on or inside their tissues. Often the eukaryotic members of the system contribute to high proportions of metagenomic sequencing reads, making it challenging to achieve sufficient sequencing depth to evaluate bacterial ecology. Stony corals are one such complex community; however, separation of bacterial from eukaryotic (primarily coral and algal symbiont) cells has so far not been successful. Using a combination of hybridization chain reaction fluorescence *in situ* hybridization and fluorescence activated cell sorting (HCR-FISH + FACS), we sorted two populations of bacteria from five genotypes of the coral *Acropora loripes*, targeting (i) *Endozoicomonas* spp, and (ii) all other bacteria. NovaSeq sequencing resulted in 67–91 M reads per sample, 55%–90% of which were identified as bacterial. Most reads were taxonomically assigned to the key coral-associated family, Endozoicomonadaceae, with Vibrionaceae also abundant. Endozoicomonadaceae were 5x more abundant in the '*Endozoicomonas*' population, highlighting the success of the dual-labelling approach. This method effectively enriched coral samples for bacteria with <1% contamination from host and algal symbionts. The application of this method will allow researchers to decipher the functional potential of coral-associated bacteria. This method can also be adapted to accommodate other host-associated communities.

Keywords: symbiosis; host-associated; metagenomics; coral; FISH; FACS; hybridization chain reaction

Introduction

It is now widely accepted that all animals and plants depend on bacteria and other microbes for their health and functioning (McFall-Ngai et al. 2013, Mueller and Sachs 2015, Sessitsch et al. 2023). In eukaryotic hosts, bacteria have been shown to play roles in processes and traits as diverse as immunity, development, digestion of food, adaptation to different environmental conditions, mate choice and other behaviours (McFall-Ngai et al. 2013, McCutcheon 2021). Corals are a notable example, as these marine cnidarians associate with several groups of microbes critical to their health and survival, including bacteria (Blackall et al. 2015, Bourne et al. 2016, Maire et al. 2022, Mohamed et al. 2023). While genomic approaches have provided an in-depth understanding of the *composition* of bacterial communities associated with reef-building corals (van Oppen and Blackall 2019), the *functions* of the bacteria within the coral holobiont (i.e. the coral animal and its associated microbiota) are still poorly understood (Sweet and Bulling 2017).

Because not all coral-associated bacteria can be isolated with conventional methods, high-quality assembled genomes derived from metagenomic analyses can be used in their absence to study whole communities. The close symbiotic relationships bacteria have with their coral hosts, in combination with the lack of host reference genomes, make it difficult to eliminate contaminating coral DNA for subsequent analyses. One approach to achieve sufficient bacterial read depth is to physically isolate them from other microorganisms and eukaryotic cells prior to DNA extraction and sequencing (Grieb et al. 2020). Fluorescence *in situ* hybridization (FISH) can assist with this enrichment process.

FISH was introduced >30 years ago as a valuable molecular tool to detect specific DNA or RNA sequences using complementary DNA- or RNA-probes labelled with fluorescent dyes (DeLong et al. 1989). Host-associated bacterial identification by standard FISH methods (i.e. the use of oligonucleotide probes labelled at either the 5′ or 3′ end with a single fluorophore) targeting ribosomal RNA (rRNA) has been explored in corals (Ainsworth et al. 2006, Apprill et al. 2012, Ainsworth et al. 2015, Damjanovic et al. 2019, Maire et al. 2023), but suffers from several limitations, such as host autofluorescence and non-specific probe binding to certain host cells and structures, that may prevent the successful detection of target organisms (Wada et al. 2016).

Autofluorescence associated with corals is the direct result of high densities of chlorophyll-containing dinoflagellates within the coral tissue and an abundance of host-derived fluorescent proteins, including green, red, cyan, and orange fluorescent protein-like molecules (reviewed in Alieva et al. 2008, Wada et al. 2016). Further, target bacterial cells could be in low abundance in some compartments of the coral animal (Maire et al. 2021) or might not be detected due to low ribosome content (Poulsen et al. 1993), or lack of permeabilization, with additional unsatisfactory signal-to-noise ratio. While some variations of FISH can amplify signal to address these challenges, such as catalysed reporter deposition

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(CARD)-FISH, they often require reagents that damage DNA (Keller and Pollard 1977) making downstream genomic analyses difficult. Instead, hybridization chain reaction (HCR)-FISH (Choi et al. 2010, Yamaguchi et al. 2015a) can boost the probe signal and transcend specific limitations ranging from low signal detection to the interference of host autofluorescence. In this approach, a specific oligonucleotide probe complementary to the rRNA target, carrying an initiator sequence, is hybridized to the cells. Next, two fluorescently labelled hairpin oligos (X1 and X2) bind subsequently in a chain reaction to the initiator sequence, thus multiplying the fluorescent signal. HCR-FISH has previously been paired with fluorescence activated cell sorting (FACS) to sort bacteria from environmental samples for single cell genomics (Grieb et al. 2020) but has yet to be applied to animals hosting complex communities of microorganisms.

In this study, we developed a combined HCR-FISH + FACS pipeline for the targeted retrieval of bacteria from coral tissues for metagenomic sequencing using the coral *Acropora loripes* as a model. Coral 16S rRNA gene metabarcoding studies have revealed that while many corals associate with several hundred and sometimes even more than a thousand different bacterial taxa (amplicon sequence variants [ASVs]) (Blackall et al. 2015), adult colonies of *A. loripes* partner with as few as 20–30 ASVs with only one or a few *Endozoicomonas* ASVs dominating the communities (Damjanovic et al. 2020). We developed our protocol using mock bacterial communities and *A. loripes* tissue to select appropriate fluorophores and FACS gates to sort two populations: 1) '*Endozoicomonas*' and 2) 'all-bacteria'.

Methods

Coral collections and tissue processing

A detailed protocol for all methods is provided in Supplemental File 1. Three *Acropora loripes* colonies were collected from Davies Reef (−18.82, 147.64; colonies Al02 and Al05 in June 2020 and Al08 in Feb. 2021) and two colonies were collected from Backnumbers Reef (−18.51, 147.15; Al11 and Al13 in December 2020) (Permit# G12/35236.1). These were transported to the Australian Institute of Marine Science's (AIMS) National Sea Simulator in Townsville, Queensland, where they were housed in outdoor mesocosms with natural light conditions and a flow-through system with seawater volume changed every two hours. The daily average temperature profile followed the average temperature profile recorded at Davies Reef weather station. Corals were maintained on a daily regimen of 0.5 *Artemia* nauplii ml^{-1} and 2 000 cells ml^{-1} of a mixed-species microalgae solution. In July 2021, 2–3 cm fragments were cut from five different locations of each coral genotype without removing the mother colony from the aquarium (to avoid mucus production). To minimise cross-contamination, nitrile gloves were discarded between each sampling location, and all collection equipment (bone cutters and forceps) was sequentially sterilized in 10% sodium hypochlorite, reverse osmosis water, 80% ethanol, with a final wash in 0.22 µm filtered seawater as described in Damjanovic et al. (2020).

Coral tissue was separated from the skeleton with minimal contamination using a divalent cation removal approach for tissue detachment (Domart-Coulon and Ostrander 2015). Coral fragments were rinsed briefly with filter-sterilized (0.22 µm membrane) artificial seawater free of calcium (CafASW, see Supp. File 1 for recipe) (Gates and Muscatine 1992, Frank et al. 1994, Domart-Coulon et al. 2004) to remove loosely associated microbes and transferred to a 50 ml polypropylene tube with ∼10 ml of CafASW. Each sample was incubated for 7 days at 4°C on a rotary mixer in CafASW to induce detachment of cells from the skeleton (Gates and Muscatine 1992, Domart-Coulon et al. 2004, Marshall and Clode 2004, Helman et al. 2008, Reyes-Bermudez et al. 2021). After the CafASW incubation, samples were moved to room temperature (RT) and supplemented with collagenase (Type IV, Sigma, Lecointe et al. 2013) at a final concentration of 0.15% (w/v) and incubated for an additional 6 hrs at 27°C on an orbital incubator (Gates and Muscatine 1992, Gates et al. 1992, Frank et al. 1994, Helman et al. 2008, Khalesi et al. 2008). Each sample was then concentrated by centrifugation (5 250 × *g* for 30 min at RT) with supernatant discarded but cells and skeleton fragments left behind. To increase permeability of cells, samples were resuspended by vortexing in 10 ml 0.1% Triton in 3x phosphate buffered saline (PBS) and incubated at RT for 5 min. After the incubation, samples were centrifuged at 5 250 × *g* for 30 min at RT. The supernatant was discarded, and the dissociated coral tissues and skeleton fragments were resuspended in 100% ethanol. At this point, samples were shipped from AIMS to The University of Melbourne (UoM) on ice.

At UoM, samples were concentrated by centrifugation (60 min at 2 885 × *g* at 4°C), skeleton removed with sterile forceps, and cells were transferred to a 1.5 ml polypropylene tube with 1 ml 100% ethanol. This cell suspension was divided into two fractions: (i) 300 µl to be labelled with EUB-mix probe suite (degenerate primer for bacteria created by merging EUB338, EUB338-II, and EUB338-III, Daims et al. 1999; Table 1) and *Endozoicomonas*-specific probes (Bayer et al. 2013; Table 1) with HCR-FISH, and (ii) 300 µl as a negative control for HCR-FISH (no probes added). These fractions will hereafter be referred to as labelled and unlabelled, respectively. As a positive control, mock bacterial communities (MC) comprised one *Endozoicomonas* (ALE010, ALC066, or ALB032; Gotze et al. in prep) and one other cnidarian-associated bacterium (*Ruegeria* sp. (ALD015), *Roseovarius* sp. (MMSF01006), or *Vibrio* sp. (MMSF00650), (Dungan et al. 2021)) and were prepared as labelled and unlabelled fractions (Fig. 1).

HCR-FISH

To remove sampling day as a confounding factor, replicate samples for each *A. loripes* genotype and the MC were processed on different days. Labelled and unlabelled samples for HCR-FISH were pelleted by centrifuging at 4°C at 10 000 × *g* for 2 min. The supernatant was discarded, and residual ethanol was removed by drying samples in a heating block set to 40°C for up to 15 min. During this time, 50 ml of hybridization/wash buffer was prepared at a 35% formamide stringency and filtered through a 0.22 µm membrane (see Supp. File 1 for recipe).

Initiator probes were purchased dry from Sigma and resuspended in nuclease-free water to 50 µM. To the labelled fractions, 10 µl of each initiator probe was added with 170 µl hybridization buffer (final probe concentration of 2.5 µM). For the unlabelled fractions, 200 µl of hybridization buffer was added. All samples were resuspended and incubated on a rotary tube mixer at 46°C overnight (12–16 hrs). After hybridization, the samples were centrifuged at 12 000 × *g* for 5 min to pellet cells and supernatant discarded. Non-specifically bound or unbound probes were removed by resuspending the samples in 500 µl of hybridization (now wash) buffer (warmed to 48°C) and incubating at 48°C for 30 min on a rotary tube mixer. During this incubation, 20 ml of amplification buffer was prepared (Supp. File 1) and filtered through a 0.22 µm membrane. Amplifier probe (Table 2) stocks were kept at 100 µM in milli-Q (MQ) water. For each labelled sample, 1 µl of stock probe

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Table 1. Initiator probes for HCR-FISH (Sigma-Aldrich). Underlined portion is the initiator sequence that the amplifier probe binds to along with a five-adenosine linker sequence. The %FA (formamide) column lists the suggested formamide percentage in the hybridization/washing buffer.

Probe	Sequence (5′-3′)	Target	%FA	Reference
Endo663-H	CCGAATACAAAGCATCAACGACTAGAAAAAA GGAAATTCCACACTCCTC	*Endozoicomonas* spp.	35	(Bayer et al. 2013, Yamaguchi et al. 2015a)
Endo736-H	CCGAATACAAAGCATCAACGACTAGAAAAAA GTCAGTGTCAGACCAGAG	*Endozoicomonas* spp.	35	(Bayer et al. 2013, Yamaguchi et al. 2015a)
EUBMix338-R	TACGCCCTAAGAATCCGAACCCTATGAAAAA GCWGCCWCCCGTAGGWGT	All bacteria	0–50	(Daims et al. 1999, Yamaguchi et al. 2015a)

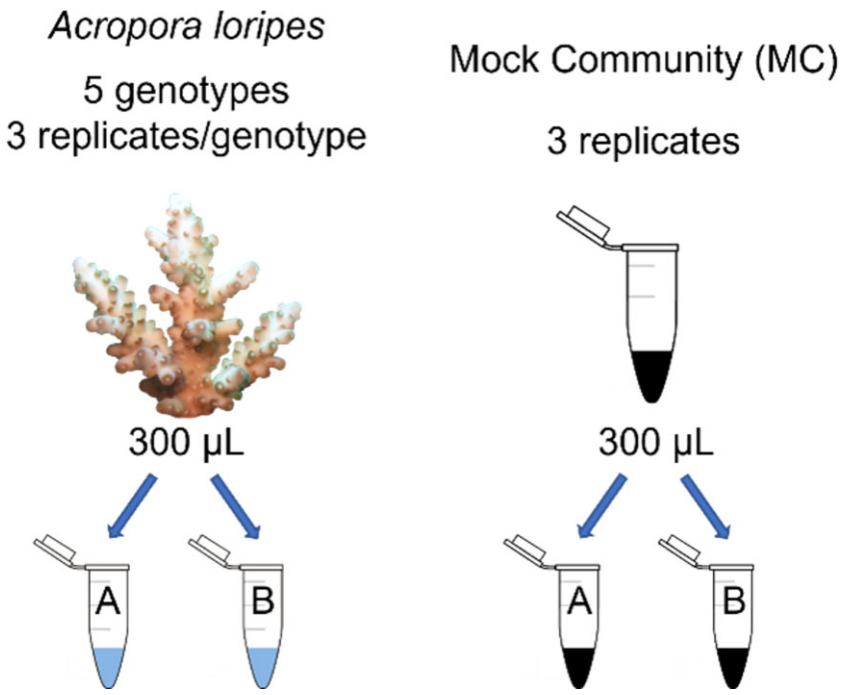


Figure 1. Setup for *A. loripes* (left) and MC (right) samples where A was **labelled** with EUBMix338 and Endo probes using HCR-FISH, and B was an **unlabelled** negative control for HCR-FISH. Replicate samples for each coral genotype and MC were processed on three separate days. The MC comprised *Endozoicomonas* and one other cnidarian-associated bacterium (*Ruegeria*, *Roseovarius*, or *Vibrio*).

Table 2. Amplifier probe sequences (Choi et al. 2010, Yamaguchi et al. 2015a) ordered from biomers net with fluorophores attached on the 5′ and 3′ end for the amplifiers 1 and 2, respectively. Lowercase letters represent stem structure of amplifier probe. Underlined sequence of H1 and R1 are complementary to the initiator sequences of initiator H and initiator R, respectively. The amplifier H pair was labelled with Atto390. Amplifier R sequences were labelled with Atto550. The emission of the selected fluorophores falls outside of the range of autofluorescence for coral and algal cells.

Probe	Sequence 5′–3′
H1	TCTAGTCGTTgatgctttgtattcggCGACAGATAAccgaatacaaagcatc
H2	ccgaatacaaagcatcAACGACTAGAgatgctttgtattcggTTATCTGTCG
R1	CATAGGGTTCggattcttagggcgtaGCAGCATCAAtacgccctaagaatcc
R2	tacgccctaagaatccGAACCCTATGggattcttagggcgtaTTGATGCTGC

was added to 49 µl of amplification buffer for each amplifier probe in the pair (i.e. H1 and H2) for a final concentration of 2 µM. Each amplifier probe was then heated to 95°C for 90 s using a hot block and kept at 25°C for 30 min prior to combining pairs. This resulted in a final concentration of 1 µM for each probe.

After washing, samples were centrifuged at 15 000 × *g* for 2 min to pellet cells. The supernatant was discarded, and all samples were resuspended in 200 µl amplification buffer (no probe). After 5 min at RT, cells were pelleted via centrifugation (15 000 × *g* for 2 min) with supernatant discarded. Labelled samples were resuspended in 200 µl of amplification buffer with the amplifier probe pair (100 µl from each R and H), whereas unlabelled samples were resuspended in 200 µl amplification buffer (no probe). All samples were incubated on a rotary tube mixer at 46°C for 3 h in the dark.

After incubation, cells were centrifuged (15 000 × *g* for 2 min). After discarding the supernatant, samples were resuspended in 200 µl of amplification buffer (no probe) for 30 min at 4°C to wash away any unbound amplifier probers and prevent probe dissociation. Prior to FACS, samples were centrifuged (15 000 × *g* for 2 min), supernatant removed, resuspended in 200 µl of MQ water, then filtered through a pluriStrainer Mini 40 µM (pluriSelect Life Science, Germany) as to remove any clumps that might clog the FACS.

FACS

Processed *A. loripes* samples were sorted on a FACS Aria III (70 µm nozzle/70 psi)/FACS Diva version 8 software (Becton Dickinson

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(BD), Franklin Lakes, NJ). Bacteria from unlabelled and fluorescent probe-labelled MC samples were used to set and confirm the cytometer scatter and fluorescence parameters and to establish gating strategies for sorting *A. loripes*-derived bacteria (Fig. S1). Briefly, bacteria were initially resolved from debris and instrument noise (not P1 gate) and single bacterial particles were separated from aggregates using the P2 gate. The P2 gate included a combination of measurements for peak particle size (forward scatter height (FSC-H) and forward scatter width (FSC-W)) or time of flight (side scatter (SSC) and forward scatter (FSC)) (Fig. 2A-C; F-H). These gates were used to select bacteria (exclude large coral or algal cells). FSC measures light scattered by particles and is directly related to the relative size. Relatively large particles, such as coral and algal cells generate higher FSC signals than bacteria. SSC is a measure of relative complexity or granularity—the more complex the internal structure of a particle, the higher this signal. Bacteria are smaller and less complex than eukaryotic cells. Unlabelled bacteria were used to set fluorescence negative boundaries in the EUB-R (Atto550) and Endo-H (Atto390) detectors, with emissions detection ranges of 582+/-7.5 nm and 450+/-20 nm, respectively (Fig. 2D-E). Bacteria from *A. loripes* labelled with Atto550 only EUB-R+/Endo-H- ('all-bacteria', P3) or double positive for Atto550 and Atto390 (EUB-R+/Endo-H+ ('*Endozoicomonas*', P5) were resolved from unlabelled particles and sorted at a rate of under 5 000 events per second into 1.5 ml tubes. Collected fractions containing between 1000 and 300 000 particles were used for subsequent metagenomics (Fig. 2I and J). FlowJo v10 (BD) was used to analyse cytometry data post-acquisition.

Sorting validation by microscopic analysis

For imaging, sorted cells were concentrated to a final volume of 30 µl by centrifugation at 4°C (5 000 × *g*; 5 min), removal of supernatant, and resuspension in MQ water. A volume of 10 µl of concentrated cell suspension was placed onto 18 well flat µ-slides coated with poly-L-lysine (Ibidi). Samples (unsorted, 'all-bacteria', and '*Endozoicomonas*' populations) were visualized on a Nikon A1R confocal laser scanning microscope at the Biological Optical Microscopy Platform (UoM) with channels for brightfield, 561 nm excitation, and 405 nm excitation.

Metagenomics preparation and data processing

Cells were pooled by genotype for each of the sorted populations by combining 500 cells from each replicate, resulting in 10 samples. Pooled populations were subjected to multiple displacement amplification (MDA) using a REPLI-g Single Cell Kit (QIAGEN) following manufacturer instructions. Following MDA at 30°C for 8 h, the DNA polymerase was inactivated, and amplified DNA was stored at −20°C. Each sample was quality checked prior to library preparation. Samples were sequenced across two lanes of a NovaSeq 6000 SP 2 × 150 bp flowcell (Illumina, San Diego, CA) at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia).

All data analysis was completed on the Melbourne Research Cloud on a virtual machine with 36vCPUs, 1.5 TB RAM, and 8 TB volume storage, following the approach of Tandon et al. (2023). Raw demultiplexed fastq files were transferred to the virtual machine; data from all samples were merged by read direction and sorted population ('all-bacteria' or '*Endozoicomonas*') following a co-assembly approach. Co-assembling samples together has the advantage of creating one single reliable baseline on which all samples can be easily compared. Paired-end reads were quality checked using FASTQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were trimmed with trimmomatic v0.36 (Bolger et al. 2014) with the following parameters: CROP:145, LEADING:30, HEADCROP:10, MINLEN:120. Trimmed reads were mapped to the *A. loripes* draft genome (Salazar et al. 2022) using bowtie2 v2.4.2 with default settings (Langmead and Salzberg 2012, Langmead et al. 2019). Unmapped paired-end reads were extracted using samtools v1.16.1 (Li et al. 2009). Assembly was performed using MegaHIT v1.2.9 (Li et al. 2015) with kmers 21,33,55,77,99 and a minimum contig length of 2 000. Metaquast v5.0.2 (Mikheenko et al. 2016) was used to extract summary data. Reads were aligned to the contigs with bbwrap (sourceforge.net/projects/bbmap/) to calculate contig coverage using default settings. Contigs were assigned taxonomy using the NCBI non-redundant (NR) database and genome taxonomy database (GTDB) with CAT v5.2.3 (von Meijenfeldt et al. 2019) which uses prodigal v2.6.3 (Hyatt et al. 2010) and DIAMOND v2.0.6 (Buchfink et al. 2015).

Host-filtered microbial reads underwent classification against bacterial genomes using Kraken 2 (Wood et al. 2019, Lu et al. 2022). The classification report was then used by Bracken (Lu et al. 2017) for species abundance estimation, which provides estimated reads per species in the sample. Bracken output files were then passed to KrakenTools (Lu et al. 2022) and Pavian (Breitwieser and Salzberg 2020) for visualization of the data via Krona plots.

Results and Discussion

HCR-FISH + FACS

Traditional in-solution FISH (Hugenholtz et al. 2001) was used in preliminary trials during the development of this method. However, we were unable to sort pure labelled bacteria from some autofluorescent coral cells as evidenced by the presence of mixed populations, observed by confocal laser scanning microscopy post FACS. To combat this issue, we successfully employed HCR-FISH (Yamaguchi et al. 2015a,b, Grieb et al. 2020) to label and sort two populations of bacteria in coral tissue homogenates from *A. loripes*. This study represents the first application of HCR-FISH in-solution for the enrichment of bacteria from holobiont samples, in this case corals. To date, HCR-FISH has been used to overcome high autofluorescence to visualize bacteria in tissue sections in one coral study (Wada et al. 2022), but it has also been applied to anemones (Goffredi et al. 2021) and the bobtail squid (Nikolakakis et al. 2015, Moriano-Gutierrez et al. 2019). CARD-FISH has been used on histology sections to better resolve bacteria within autofluorescent shallow (Chiu et al. 2012, Neave et al. 2016) and deep-water (Thompson and Gutierrez 2021) coral tissues, but this approach requires reagents that damage DNA (Keller and Pollard 1977). CARD-FISH would therefore not be suitable for post-labelling metagenomic applications.

Previous FACS work in corals have prepared cell suspensions by mechanical disruption after incubation in calcium free media (Rosental et al. 2017, Levy et al. 2021). Here, the dissociation of cells was accomplished using enzymatic tools rather than mechanical approaches to fully dissociate cells and reduce bacterial contamination. Future applications of this method should consider quantifying bacterial load via quantitative or digital PCR to compare the efficiency of a mechanical versus enzymatic approach. This step could be improved further by visualizing cells after dissociation to compare methods. Sorting of FISH-labelled bacterial cells has previously been done using standard FISH on mixed bacterial cultures (Wallner et al. 1997), sludge from a bioreactor (Miyauchi et al. 2007), or marine sediment (Kalyuzhnaya et al. 2006), CARD-FISH on seawater samples (Sekar et al. 2004), or HCR-FISH on

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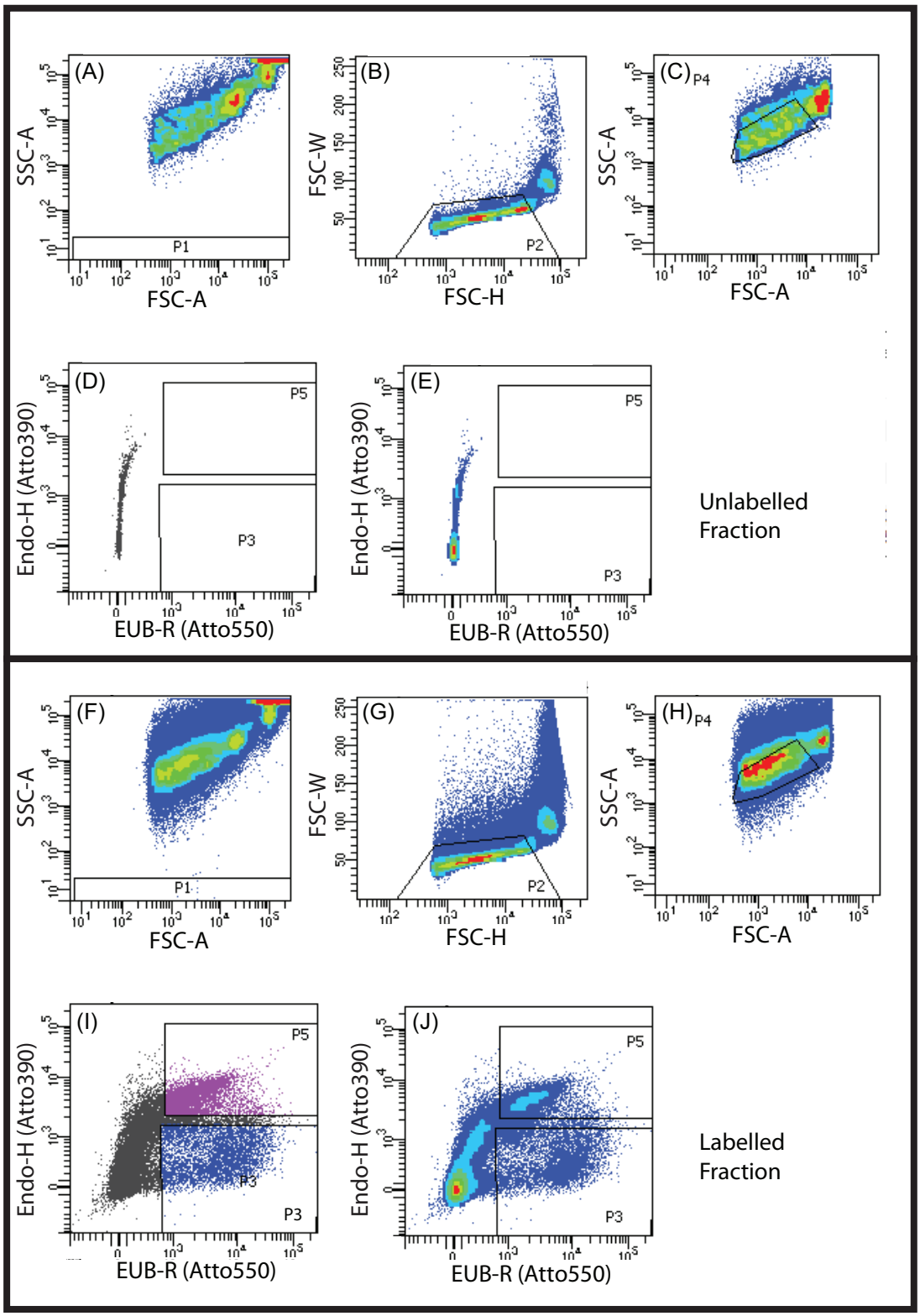


Figure 2. FACS sorting profiles for unlabelled (A-E) and labelled (F-J) *A. loripes* tissue homogenate (genotype Al08). First, background noise (P1) from the FACS was excluded (A, F). Large coral and Symbiodiniaceae cells were then identified by forward (B, G) and side (C, H) scatter and excluded through sequential gating. Fluorescent channels were used to identify autofluorescence of the remaining events in the unlabelled fraction in the 582 nm (D-E x-axis) and 450 nm (D-E y-axis) emissions. Events in the labelled fraction that surpassed this baseline autofluorescence in both axes were collected as the dual labelled 'Endozoicomonas' population (P5) and those that had greater signal on the x-axis only were collected as the single labelled 'all-bacteria' population (P3) (I-J).

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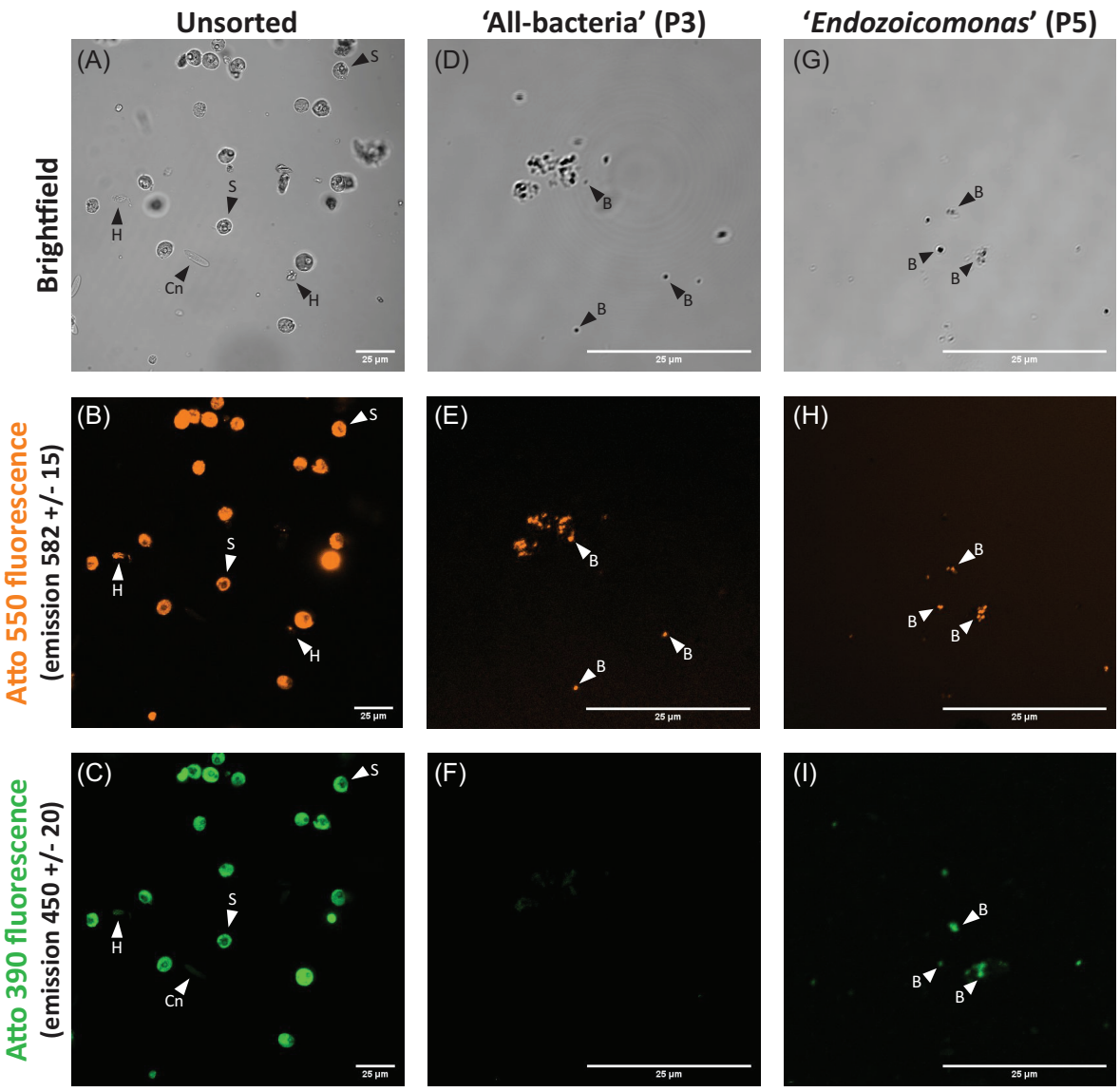


Figure 3. A labelled A. loripes sample (colony Al13) prior to sorting with FACS (A–C) or sorted cell populations that were single ('all-bacteria', D–F), or dual ('Endozoicomonas', G–I) labelled. Each sample was visualized on a Nikon A1R confocal laser scanning microscope with channels for brightfield (A, D, G), 561 nm excitation (B, E, H), and 405 nm excitation (C, F, I). S=Symbiodiniaceae, Cn=host cnidocyst, H=uncharacterised host cell, B=bacteria. All scale bars are 25 µm.

marine phytoplankton samples (Grieb et al. 2020). These studies have sequenced PCR products of specific genes like the 16S rRNA gene from sorted cells. Whole genome sequencing has been attempted from FISH labelled and sorted cells, but the recovered genomes suffered from low completeness (Podar et al. 2007, Yilmaz et al. 2010).

Using physical parameter properties FSC and SSC, and fluorescent probes, we established conditions to differentiate bacteria from non-bacterial particles and to distinguish and separate '*Endozoicomonas*' (dual labelled) and 'all-bacteria' (labelled with EUBMix338 only) populations (Fig. 2; Fig. S1). FACS is a highly sensitive technique for detecting and measuring fluorescence signals from individual cells or particles with high precision and resolution. So, while the sorted populations were visually free of host cells and debris (Fig. 3), the sensitivity of confocal microscopy may be limited compared to FACS due to factors such as background noise and detection efficiency. Because coral tissue is known to exhibit non-specific binding of the EUBMix338 probe (Wada et al. 2016), we also trialled HCR-FISH+FACS with a nonsense probe, NONEUB338, which has a nucleotide sequence complementary to the nucleotide sequence of probe EUBMix338 (Christensen et al. 1999). These trials were inconclusive because the NONEUB338 initiator sequence can bind to the 16S rRNA gene in the bacterial DNA and, with HCR-FISH amplification, the signal is sufficient for FACS detection. Future studies should consider ways to address non-specific binding with HCR-FISH+FACS, such with the addition of a blocking reagent (Yamaguchi et al. 2015a).

Metagenomics

Merging all data for the '*Endozoicomonas*' and 'all-bacteria' populations resulted in 326 M and 307 M read pairs, with 313 M and 297 M read pairs remaining after quality filter and trimming by

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trimmomatic, respectively. Of these, only 0.68% of reads in the '*Endozoicomonas*' and 0.01% in the single-labelled 'all-bacteria' population aligned to the reference host *A. loripes* genome (Salazar et al. 2022). Of the total reads, 169.3 M and 181.5 M from the '*Endozoicomonas*' and 'all-bacteria' populations, respectively, were used to assemble contigs.

A total of 4785 and 4594 contigs were assembled from the 'all-bacteria' and '*Endozoicomonas*' populations, respectively (Table 3). For the 'all-bacteria' contigs, 822 were taxonomically identified as eukaryotes (12.0% reads), 2033 contigs were unidentified (18.1% reads), and 1930 contigs were identified as bacteria (69.8% reads). Of those bacterial contigs, the most abundant families were Endozoicomonadaceae (584 contigs, 9.1% reads), Sporolactobacillaceae (160 contigs, 1.1% reads), Peptostreptococcaceae (74 contigs, 1.9% reads), and Vibrionaceae (36 contigs, 0.2% reads). However, the contigs were generally short (Endozoicomonadaceae mean±SD 5 459±3790 bp), with highly variable fold coverage (Endozoicomonadaceae ranges 5–37 744x coverage, mean±SD 1188±3658x). There were only nine symbiodiniacean and four cnidarian contigs, highlighting the success of the sorting in enriching samples for bacteria. For the '*Endozoicomonas*' contigs, 196 were identified as cnidarian (0.4% reads), three were apicomplexan (0.007% reads), and 18 were Symbiodiniaceae (0.1% reads). Of the remaining contigs, 890 were unidentified (12.0% reads), one contig (0.1% reads) was identified as a virus, and 3 055 contigs were identified as bacteria (75.4% reads; Table 3). Of the bacterial contigs, by far the most abundant family was Endozoicomonadaceae with 1298 contigs (47.8% reads). These contigs averaged 7705±7346 bp (mean±SD), had 75 081–5x fold coverage (mean±SD 2538 ± 5429x) and total length of 10 Mbp.

While Endozoicomonadaceae reads were still present in the 'all-bacteria' population, they were ~5-fold less abundant than in the '*Endozoicomonas*' population (9.1% reads in 'all-bacteria' versus 47.85% reads in '*Endozoicomonas*'; Supp. File 2), highlighting the success of the dual-labelling approach. Because *Endozoicomonas* spp. are highly abundant in *A. loripes* (Damjanovic et al. 2020), by concentrating this taxon in one population of sorted cells we hoped to provide greater read depth of rare or novel taxa. This is apparent in the 'all-bacteria' population, where 52% of the bacterial reads (Supp. File 2) were unidentified, suggesting that they represent novel environmental microbes not yet present in the GTDB reference database. This is compared to only 22% of bacterial reads assigned as no support in the '*Endozoicomonas*' population (Supp. File 2).

The low level (<1%) of coral and algal symbiont contamination in the metagenomic sequence data for our coral tissue samples is unique. Previous work to enrich prokaryotes from coral samples prior to metagenomic sequencing (Table 4) have applied percoll gradient fractionation (Wegley et al. 2007, Dinsdale et al. 2008, Vega Thurber et al. 2009, Littman et al. 2011), differential centrifugation (Keller-Costa et al. 2021, Keller-Costa et al. 2022), or sequential filtration (Robbins et al. 2019). These approaches however have been unsuccessful in providing researchers with metagenomic data dominated by bacterial reads, in part because percoll fractionation to enrich for bacteria will also capture host mitochondria (Wegley et al. 2007), and residual host DNA can contaminate centrifugation and filtration strategies. When coral tissue has been sequenced more recently without enrichment for prokaryotes, reads from the eukaryotic populations accounted for over 90% of the reads in most cases (Roach et al. 2020, Rosales et al. 2022). Our enrichment method implementing HCR-FISH prior

Table 3. Stats from metagenomic data analysis for the 'Endozoicomonas' and 'all-bacteria' populations.

Sorted population	Raw reads (M)	Reads after trimmomatic (M)	Read pairs after removing *A. loripes* (M)	Reads used to assemble contigs (M)	Contigs	Total contig length Mbp	Max/Avg contig length (bp)	N50	Avg. fold coverage	#contigs no hit—read pairs (M)	#contigs euk—read pairs (M)	#contigs bacteria—read pairs (M)	# of Endozoicomonadaceae contigs—read pairs (M)
'*Endozoicomonas*' (P5)	326	313	311	169.3	4 594	27.4	165 029/5 972	7 643	1 434	890–20.3	648–21.1 (18 Symbiodiniaceae; 196 Cnidarian)	3 055–127.6	1 298–81.0
'All-bacteria' (P3)	307	297	297	181.5	4 785	24.9	102 783/5 207	6 059	1 100	2 033–32.9	822–21.9 (9 Symbiodiniaceae; 4 Cnidarian)	1 930–126.7	584–16.6

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Table 4. Details from existing stony coral metagenomics work where the primary sequencing target was coral-associated microbes. ∗The % of total reads that were classified as bacterial from the authors chosen reference database†. Where studies collected metagenomes from corals and other substrates (i.e. seawater, algae), only the data from the corals is included.

Coral Species	Geographic location	Target	Enrichment method	Classified bacterial sequences∗ (% of total reads)	Classification database†	Sequencing Platform	Reference
Porites asteroides	Caribbean	All microbes	Percoll fractionation	1.5%	SEED	454 Pyrosequencing	(Wegley et al. 2007)
Porites compressa	Hawaii	All microbes	Percoll fractionation	0.6–1.0%	SEED	454 Pyrosequencing	(Dinsdale et al. 2008, Vega Thurber et al. 2009)
Acropora millepora	Magnetic Island, GBR	All microbes	Percoll fractionation	0.2–0.9%	SEED	454 Pyrosequencing	(Littman et al. 2011)
Platygyra carnosa	Lamma Island and Crescent Bay, South China Sea	Bacteria	Percoll fractionation	0–40%	NCBI NR	Illumina HiSeq	(Cai et al. 2017)
Porites lutea	Orpheus Island, GBR	Bacteria and archaea	Sequential filtration	0.2–15%	Greengenes/GTDB	Illumina HiSeq2500 (2×150 bp)	(Robbins et al. 2019)
Pseudodiploria strigosa Orbicella faveolata	Curaçao, Caribbean	All microbes	NA	0.93%	SEED	Illumina MiSeq (2×300 bp)	(Roach et al. 2020)
Acropora tenuis Goniastrea minuta Pocillopora verrucosa Pocillopora meandrina	Xiane Reef, South China Sea	All microbes	NA	<20% in healthy corals; 33–79% in bleached corals	NCBI NR	Illumina HiSeq (2×150 bp)	(Sun et al. 2020)
Pseudodiploria strigosa	Bermuda	Bacteria in coral surface mucus layer (SML)	Modified syringe used to collect SML only	50.2% average	MG-RAST (Refseq/SEED)	Illumina MiSeq	(Lima et al. 2022)
Stephanocoenia intersepta, Diploria labyrinthiformis, Dichocoenia stokesii, and *Meandrina meandrites*	Looe Key and East Washerwoman Reef, Florida Keys	All microbes in tissue and mucus	NA	19.2% average (skewed by one sample); ranged 0.9–67.3%	GTDB	Illumina HiSeq 4000	(Rosales et al. 2022)
Porites lutea Goniastrea edwardsi	Abu Shoosha Reef, Red Sea	Skeleton bacteria	Fragmentation to remove tissue	Not provided	GTDB	Illumina HiSeq 4000 (2×151 bp)	(Cardenas et al. 2022)
Porites lutea Isopora palifera	Heron Island, GBR	Skeleton bacteria and archaea	Coral tissue was removed from the fragments using a Waterpik and sterile seawater; the remaining skeletons were snap frozen.	Not provided	GTDB	DNBSeq	(Tandon et al. 2023)
270 samples from *Pocillopora*, *Porites*, and *Millepora*	Tara Pacific expedition	All microbes	NA	Not provided	Centrifuge v1.0.3, NCBI NR	Illumina NovaSeq6000 or HiSeq4000	(Belser et al. 2023, Hochart et al. 2023, Lombard et al. 2023)
Acropora loripes	Davies and Backnumbers Reef, GBR	Tissue Bacteria	HCR-FISH + FACS	72.6% average	GTDB	Illumina NovaSeq6000 SP (2×150 bp)	This study

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to FACS is the first to average >70% bacterial reads from coral tissue samples.

When completing metagenomic sequencing on compartments of the coral holobiont that do not include tissue, contamination by host or other eukaryotic reads is less prominent. Tandon et al. (2023: effectively assembled 393 high-quality metagenome assembled genomes (MAGs) from coral skeleton fragments by sequencing samples on individual lanes; in only two samples were there >45% host reads. Cardenas et al. (2022) were able to achieve metagenome sequences that were dominated (~75%) by bacterial reads when working with skeletal material. However, to compare bacterial communities between skeleton and tissue they had to use 16S rRNA gene metabarcoding. Metagenomics of the surface mucus layer (SML) of a Caribbean coral species contained ~50% of reads that were identified as bacteria with no additional enrichment required (Lima et al. 2022).

MDA (Dean et al. 2002) was used in this study to obtain sufficient DNA from sorted bacteria. However, it has drawbacks such as amplification bias (Ahsanuddin et al. 2017), poor uniformity, errors and artifacts, low genome coverage, inability to address all variant classes, low accuracy, poor reproducibility, and/or complex protocols that are difficult to automate or scale. Reads generated in this study were heavily skewed toward some regions of bacterial genomes resulting in orders of magnitude differences in coverage and an inability to generate MAGs, which is likely a result of uneven amplification during the 8 hrs of MDA.

Ideally, researchers should aim to collect enough cells so that amplification is not necessary. In these cases, DNA can be extracted with low biomass-input methods (Bramucci et al. 2021). When this is not possible, a potential alternative to MDA is primary template-directed amplification (PTA) (Gonzalez-Pena et al. 2021). PTA is an isothermal whole genome amplification method that reproducibly captures near-complete genomes of single cells while suppressing the formation of experimental artifacts such as chimeric molecules and non-specific priming (Telenius et al. 1992). PTA may be performed directly on DNA from single cells (collected by FACS, microfluidic or other methods), multiple cells, or ultra-low inputs of DNA (>4 pg– 10 ng). Future applications of this method to enrich complex communities for bacteria prior to metagenomics should use caution with MDA and amplify for the shortest duration of time required to get sufficient DNA for sequencing.

Conclusion

Our findings show that HCR-FISH + FACS is a substantially improved method to obtain host-associated bacteria for metagenome sequencing, where standard metagenomic techniques do not work for low-abundant bacteria due to noise from more common species (typically the host species).Our method makes the analysis of uncharacterized microbes simpler and more accessible, and provides researchers with an enhanced platform to address the grand challenge of deciphering the functions of host-associated bacteria in symbiosis. As our method can be implemented in holobionts other than coral, we believe that this innovative approach holds promise for advancing the field of microbial ecology.

Author contributions

Ashley M. Dungan (Conceptualization, Formal analysis, Methodology, Writing – original draft), Kshitij Tandon (Formal analysis, Writing – reviewing and editing), Vanta Jameson (Formal analysis, Resources, Writing – reviewing and editing), Cecilie Ravn Gotze (Methodology, Writing – reviewing and editing), Linda L. Blackall (Conceptualization, Funding acquisition, Supervision, Writing – reviewing and editing), and Madeleine J. H. van Oppen (Conceptualization, Funding acquisition, Supervision, Writing – reviewing and editing)

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Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

Conflict of interest: The authors declare no conflicts of interest.

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Data availability

The data sets generated and analysed during the current study are available under NCBI BioProject ID PRJNA1036684.

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