

Fine-scale mapping of physicochemical and microbial landscapes clarifies the spatial structure of the coral skeleton microbiome

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Abstract

The coral skeleton harbours a diverse community of bacteria and microeukaryotes exposed to gradients of light, O₂ and pH, but how such physicochemical gradients affect the coral skeleton microbiome remains unclear. We employed chemical imaging of O₂ and pH, hyperspectral reflectance imaging and spatially explicit taxonomic and functional microbiome characterisation to explore links between the skeleton microenvironment and microbiome in the reef-building corals *Porites lutea* and *Paragoniastrea australensis*. The physicochemical environment was more stable in the deep skeleton and the diversity and evenness of the bacterial community increased with depth, suggesting that the microbiome was stratified along the physicochemical gradients. The bulk of the coral skeleton was a low O₂ habitat, whereas pH showed a great variation with depth between pH 6 and 9. Physicochemical gradients of O₂ and pH of the coral skeleton correlated with the β-diversity of the bacterial communities. Skeletal layers that showed O₂ peaks had a higher relative abundance of endolithic algae, reflecting a strong link between the abiotic environment and the microbiome composition. Altogether, these results link the physicochemical, microbial and functional landscapes of the coral skeleton and provide new insights into the involvement of skeletal microbes in the holobiont metabolism.

Introduction

A dynamic endolithic (rock-dwelling) microbiome lives within the coral skeleton along steep gradients in physicochemical properties (Kühl et al. 2008; Ricci et al. 2019). These gradients change over the skeleton's vertical axis from the surface towards the interior of the coral colony and might determine the stratification of the endolithic community, where O₂ and pH gradients can be linked to heterotrophic and autotrophic metabolism of microbiome members (Bellamy and RiSK 1982; Shashar and Stambler 1992; Kühl et al. 2008). For instance, the coral skeleton is mainly an anoxic environment (Kühl et al. 2008) but there is some evidence indicating that skeletal layers of branching species hosting a high abundance of endolithic green algae (*Ostreobium* spp.) can reach O₂ levels as high as 200% air saturation in light (Bellamy and RiSK 1982). These abrupt changes in the physicochemical environment can be linked to the metabolism of certain members of the microbiome such as endolithic algae and *Cyanobacteria* (Magnusson et al. 2007; Kühl et al. 2008), but whether these physicochemical changes, in turn, affect the presence and abundance of other microbes (including endoliths) remains to be investigated.

The calcium carbonate (CaCO₃) matrix of the skeleton of living scleractinian corals is colonized by endolithic communities of eukaryotes and prokaryotes (Marcelino and Verbruggen 2016; Marcelino et al. 2017; Pollock et al. 2018; Ricci et al. 2022). Endolithic, coenocytic green algae in the genus *Ostreobium* are among the most conspicuous eukaryotes found in this environment (Marcelino and Verbruggen 2016; Del Campo et al. 2017; Iha et al. 2021; Ricci et al. 2021; Tandon et al. 2022), and they often form a distinct green band positioned one to a few mm below the coral tissue. The ability of *Ostreobium* filaments to dissolve and drill into the CaCO₃ matrix makes them ubiquitous colonizers of the coral skeleton (Ralph et al. 2007). Fungi also excavate the CaCO₃ matrix (Le Campion-Alsumard et al. 1995; Priess et al. 2000) and can, in some cases, attack the endolithic algae, turning green layers into black (Priess et al. 2000). The marine plant parasite *Labyrinthula* is one of the most abundant eukaryotes in common coral species from the Great Barrier Reef and its distribution within the skeleton might be linked to interactions with the endolithic algae (Ricci et al. 2021). The bacterial community of the coral skeleton is very diverse (Sweet et al. 2011; Marcelino and Verbruggen 2016; Marcelino et al. 2018; Pollock et al. 2018; Ricci et al. 2022), its composition is influenced by the skeleton architecture (Ricci et al. 2022) and it can colonize shallower as well as deeper skeletal layers (Ralph et al. 2007; Ricci et al. 2021). Some bacteria, like the order *Myxococcales*, and genera *Spirochaeta* and *Endozoicomonas*, show persistent association with the coral skeleton (Marcelino and Verbruggen 2016; Pollock et al. 2018; Ricci et al. 2022) and are thought to be involved in aiding host homeostasis through nutrient cycling and antibiotic productions (Lawler et al. 2016; Neave et al. 2016; Pollock et al. 2018; Ricci et al. 2022). However, we still know very little about factors that shape the coral skeleton microbiome with increasing depths in the skeletal matrix.

The coral skeleton exhibits a distinct physicochemical environment (Ricci et al. 2019) that is strongly shaped by the physical properties of the porous CaCO₃ matrix (Wu et al. 2009) and the incident solar irradiance (Magnusson et al. 2007). While far-red light (> 700 nm) penetrates the coral tissue and CaCO₃ skeleton efficiently, visible wavelengths (400–700 nm) are strongly attenuated at the level of the green *Ostreobium* layer to < 0.1 to 15% of the incident irradiance at the coral tissue surface (Magnusson et al. 2007). The light attenuation is due to a combination of pigment absorption and strong scattering in the CaCO₃

matrix that leads to a diffuse light environment as a function of the distinctive skeletal architecture of each coral species (Enríquez et al. 2005; Terán et al. 2010; Marcelino et al. 2013). While the skeleton is mainly an anoxic environment, O₂ is produced both in the coral tissue and in the green *Ostreobium* layer during the daytime and diffuses across the porous skeletal matrix (Kühl et al. 2008). Photosynthetic CO₂ fixation contributes to changes in pH in the green skeletal layer, which in the coral *Porites compressa* shifted from ~ 7.7 in darkness to ~ 8.5 in light (Shashar and Stambler 1992). In darkness, the pH shifts to pre-illumination values mainly because of CO₂ production through respiration (Shashar and Stambler 1992), but very few spatially resolved pH measurements have been done in coral skeletons (Shashar and Stambler 1992; Reyes-Nivia et al. 2013). There is now increasing evidence for various microbial processes involved in carbon, nitrogen, sulphur, and phosphorus transformations in the coral skeleton (Ferrer and Szmant 1988; Yang et al. 2016; Sangsawang et al. 2017; Yang et al. 2019; Moynihan et al. 2021), but quantification of such processes is rare and it remains unknown how the functions of the skeletal microbiome change with depth and skeletal architecture. By using a combination of hyperspectral imaging, planar optode sensors and spatially explicit microbiome characterization (by metabarcoding), the present study explores correlations between the O₂ and pH microenvironment and the composition and inferred functions of the skeletal microbiome.

Materials And Methods

Experimental design

We used the coral species *Porites lutea* and *Paragoniastrea australensis* (six specimens for each species) because their distinctive skeletal architectures influence the colony's physicochemical environment (Wu et al. 2009; Marcelino et al. 2013) and microbial community (Fordyce et al. 2021; Ricci et al. 2022). Furthermore, with the aim of reducing the variability known to influence the composition of the microbiome living in association with corals, such as colony age (Williams et al. 2015), health status (Maher et al. 2019) and spatial or temporal variability (Dunphy et al. 2019), we collected fragments of healthy, adult colonies, a maximum of 300 m apart and over a three-week period from the reef flat of Heron Island, Great Barrier Reef in January 2020.

We cut each coral skeleton into two halves (side A and B; Fig. 1). On side A, we used planar optode chemical imaging to characterize the O₂ and pH gradients over coral skeleton cross-sections, and then we used hyperspectral reflectance imaging to map the distribution of photosynthetic pigments (Fig. 1). From side B, we obtained five sequential sub-samples of 4x4x4 mm (= 64 mm³) at increasing distances from the surface of skeleton side A (Fig. 1). We assessed the prokaryotic and eukaryotic diversity in each skeletal sub-sample using 16S and 18S rRNA metabarcoding in combination with functional prediction bioinformatics analyses to infer bacterial metabolisms (Fig. 1).

Sample collection and processing

Twelve coral fragments were collected at low tide (< 1 m water depth) from the research zone of Heron Island reef flat, Great Barrier Reef (GBR; 23°44'S, 151°91'E), during January 2020. Specimens were collected using a sterile hammer and chisel and placed in sterile zip-lock polyethylene bags in seawater. Each coral skeleton was cut into two halves (side A and B; Fig. 1) using a diamond saw with a continuous flow of sterile filtered (0.22 µm) seawater (SSW). Skeleton side A was covered with black, nontoxic plasticine to prevent light and seawater penetration and left to acclimate for two days in shaded outdoor flow-through aquaria flushed with lagoon water. A 4-mm thick slice was cut perpendicular to the vertical growth axis of the colony from skeleton side B and thoroughly rinsed with SSW. The coral tissue was removed from the skeleton 4-mm thick slice using a waterpik and SSW and sterilized razor blades were used to subdivide each slice into 4 x 4 mm sub-samples (each representing a skeleton volume of 64 mm⁻³), resulting in a total of five skeletal sub-samples for each coral colony (Fig. 1). The coral skeleton sub-samples were collected and snap-frozen by immersion in liquid nitrogen and stored at -80°C until processing. Two samples of SSW (5 L each) were filtered using 0.22 µm filters (MilliporeSigma) and snap-frozen. To avoid possible cross-contamination we used sterilized razor blades and tweezers for each sub-sample and sequenced SSW and control samples taken during the DNA extraction and amplification. Afterwards, the sequences retrieved from SSW, extraction and amplification controls were removed from the dataset using the R package *decontam* (Davis et al. 2018).

Library preparation, sequencing and initial quality control

The total DNA from the snap-frozen skeletal sub-samples was extracted using the Wizard Genomic DNA Purification Kit (Promega). Extractions were also performed on SSW, seawater taken at the time of sampling, and three blanks taken during both the extraction and amplification protocols. These SSW and blanks served as controls. We used a 2-step PCR amplification, the first PCR amplified the target markers, and the second PCR added the Illumina adapters overhangs (underlined). The V5-V6 regions of the 16S rRNA were PCR amplified using the primer pairs: 784F [5'- GTGACCTATGAACTCAGGAGTCAGGATTAGATACCCTGGTA - 3'], and 1061R [5'- CTGAGACTTGCACATCGCAGCCRRACAGCTGACGAC - 3']. The 18S rRNA gene was amplified using the primer pairs: NF1 [5'- GTGACCTATGAACTCAGGAGTCGGTGGTGCATGGCCGTTCTTAGTT - 3'], and 18Sr2b [5'- CTGAGACTTGCACATCGCAGCTACAAAGGGCAGGGACGTAAT - 3'] (Porazinska et al. 2009). The first PCR round was conducted in 20 μ L reactions using the KAPA HiFi HotStart ReadyMix and 10 μ L IM of each primer, with a thermal cycling profile of 95°C for 3 min; 25 cycles of 98°C for 20 s, 60°C for 15 s, 72°C for 30 s, a final extension at 72°C for 1 min. The second PCR round was conducted in 20 μ L reactions using the GoTaq Green mix and 10 μ M of each custom-made Illumina index. The thermal cycling profile was: 95°C for 3 min; 24 cycles each at 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, a final extension at 72°C for 7 min. Amplicons of the sub-samples and controls were sequenced in the Illumina MiSeq platform (2 x 300 bp paired-end reads) at the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia.

Sequences were processed using the QIIME2 pipeline version 2020.11 (Bolyen et al. 2018). Cutadapt was used to remove primers (Martin 2011), DADA2 was used to merge forward and reverse reads, remove poor-quality sequences, perform dereplication and eliminate chimeras (Callahan et al. 2016). Taxonomy was assigned using the feature-classifier plugin in-built in QIIME2. For 16S rRNA, we used the SILVA v132 QIIME release (Quast et al. 2012) and for 18S rRNA the PR2 database (Guillou et al. 2012). The bacterial and eukaryotic phototrophic community was curated from the 16S and 18S rRNA gene datasets based on literature (Fenchel et al. 2012; Lohr et al. 2012).

Chemical Imaging

The O₂-sensitive optode sensor was prepared using 100 mg of polystyrene, 1.5 mg of indicator (PT(II) Meso-tetra(pentafluorophenyl)porphine), 1.5 mg of reference (Makrolex yellow®) and dissolved in 1 g of solvent (Tetrahydrofuran) to form a cocktail. The pH-sensitive optode sensor was prepared using 200 mg of hydrogel D4, 1 mg of indicator (lipophilic 8-hydroxy-1,3,6-pyrene-tri sulfonic acid derivate), 1 mg of reference (perylene) and dissolved in 2 g of solvent (Tetrahydrofuran) to form a cocktail. The O₂ and pH cocktails were knife-coated on dust-free polyester foils (goodfellow.com) and the final thickness of the coating was < 2 μ m.

The experimental setup was composed of a digital single-lens reflex (Canon EOS 1000D) with the NIR blocking filter removed and equipped with a Sigma 50 mm F2.8 EX DG Macro Lens. The camera lens was also equipped with emissions filter Schott 530 nm for O₂ and Schott 455 nm long-pass filters (Uqgoptics.com) for pH. As described in Larsen et al. (Larsen et al. 2011), the O₂-sensitive sensor was excited by 4 high power blue LEDs (I-peak = 445 nm, LXHL-LR3C, Luxeon, F = 340 mW at IF = 700 mA) in combination with a 470 nm short-pass filter (Blue dichroic colour filter, Uqgoptics.com), while the pH-sensitive sensor was excited by 4 high power UV LEDs (I-peak = 405 nm, LZ1-10UA05, LedEngin, F = 460 mW at IF = 700 mA) used in combination with a 405 nm band pass filter (NT43-156, Edmundoptics.com). The coral skeleton cross-sections were illuminated through the planar optodes by a Schott Leica KL 2500 LCD Cold Light Sources. All the elements of the setup were connected to a trigger box (<https://imaging.fish-n-chips.de>) and controlled through a PC using the custom-made software Look@RGB.

Each planar optode was calibrated independently in an aquarium with a constant seawater temperature of 27°C in a darkened room. The calibration range was 0-340 μ mol L⁻¹ for the O₂-sensitive planar optode and pH 6-9 for the pH-sensitive planar optodes (Supp. Figure 1). During the pH calibration, cross-sections of *P. lutea* and *P. australensis* cleared skeletons were placed on the planar optodes with the aim of accounting for the scattering properties of the skeleton of each coral species (Marcelino et al. 2013). This is of course only an approximation, as it was not possible to use the exact same fragment for calibration and measurement. For the O₂ calibration, we accounted for the scattering properties of the skeletons by subtracting the first (calibrated) image of each experiment from subsequent images. These first images were taken ~ 8 hours after the cut coral fragments were placed on the O₂-sensitive planar optodes, in complete darkness; thus, the skeletal cross-sections were assumed to be fully anoxic and any detected signal would be due to skeletal scattering. Luminescence intensities of the planar optode are

highest at anoxic conditions, which thus would result in the highest scattering. We recommend seeing Larsen et al. (2011) for detailed methods, equipment and calibration process of planar optodes in general.

All experiments were performed in a dark room at a constant temperature of 27°C to resemble the seawater temperature at the time of sampling. Groups of two or three coral halves were placed in a 4L glass aquarium on top of transparent, calibrated planar O₂ and pH-sensitive optodes, and the aquarium was placed on top of the imaging setup (Supp. Figure 2). Seawater in the aquarium was aerated with an aeration stone connected to an air pump. To impair the exchange of O₂ between the surrounding water column, the coral tissue and the coral skeletons cut surface, nontoxic Plasticine sealed the coral edges and was fixed to the optode. After this process, corals were left to acclimate overnight while fixed on the planar optode. The cut coral surface was illuminated through the optode (Supp. Figure 2) for 360 minutes followed by 240 minutes of darkness at each of the following incident photon irradiances (400–700 nm): ~8, ~21 and ~50 μmol photons m⁻² s⁻¹ using fiber-optic halogen lamps (Schott KL 2500 LCD). Irradiance levels in the experimental setup for defined lamp settings were measured using a Walz Universal Light Meter (ULM-500) equipped with a Mini Quantum Sensor (LS-C). Unless technical problems such as electricity outages did not permit carrying out the full experiment, image sequences capturing O₂ or pH dynamics across the corals cut surfaces were acquired every minute for a total of 600 images for each light setting (~8, ~21 and ~50 μmol photons m⁻² s⁻¹).

Downstream data analysis was performed using ImageJ v1.53K. Every image was split into Red, Blue, Green and Green2 RAW TIFF images. Using the ImageJ plugin *Ratio Plus* we divided the Red by the Green RAW TIFF images (R/G) for the O₂ data analysis and the Green by the Blue RAW TIFF images (G/B) for the pH data analysis. The resulting images were colour-coded using the lookup-table 'Fire' to visualize the dynamics. Then by using the curve fitting function *Curve Fitting (Exponential with Offset* for O₂ and *Straight Line* for pH), the images were calibrated according to the planar optode calibration values. By using the function *Brightness&Contrast*, the minimum and maximum displayed values of each image were set to 0 and 340 μmol/L for O₂ images and 6 and 9 for pH images. Given the low O₂ concentration we measured in the skeletons we used the function *Threshold* to only display dissolved O₂ concentration values between 0 and 15 μmol L⁻¹. In the O₂ data analysis, we used the function *Image Calculator* to remove the first image of every experiment from the subsequent images with the aim of minimizing scattering artefacts of the skeleton. We obtained O₂ and pH values from every image by defining five Regions of Interest (ROI) of ~4x4mm² from shallower to deeper skeletal layers and measuring their value using the function *Measure*. These ROIs were chosen according to the sub-samples collected along the vertical axis of the coral skeleton used in the characterization of the 16S rRNA and 18S rRNA genes. The obtained values are presented in the manuscript and were used for statistical analysis in combination with molecular data.

Hyperspectral Imaging

Hyperspectral imaging of reflected light was performed on the vertical cross-section of the skeleton of three *Po. lutea* and three *Pa. australensis* half colonies submerged in seawater (27°C and salinity = 35). The measurements were done with a hyperspectral camera system (Snapscan VNIR, Imec, Belgium) using automatic dark signal correction and normal halogen lamps without a heat filter for illumination of the coral cross-section. White referencing was done using a 95% reflectance tile (Imec, Belgium) placed at the same distance, position and light field as the coral cross-sections. Hyperspectral image analysis (and export of reflectance spectra from particular areas in the coral cross-sections) was done with the Snapscan operating software (Imec, Belgium). Hyperspectral image cubes obtained for the coral cross-sections were normalized with the white reflectance standard to obtain hyperspectral image cubes in units of % reflectance for the coral cross-sections (see also Kühl et al. 2020). Using the Imec Spectral Angle Classifier in the system software, we could highlight areas with identical spectral properties over the coral cross-sections. For this, we trained the classifier by defining small areas of the cross-section with particular spectral features, where each selection was treated as a separate class by the classifier. The classifier analysis yielded false color-coded maps of the coral cross-sections highlighting regions with similar spectral properties.

Predicted functional abundances based on 16S rRNA gene

Functional profiles of the total skeletal microbial community were extrapolated by a conservative approach using a combination of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; Douglas et al. 2020) and Tax4Fun2 (Wemheuer et al. 2020). The ASV abundance table and sequences of the whole community and phototrophs were used as input for PICRUSt2 with the following parameters, *-in_traits* KO and *-stratified*. Results from Tax4Fun2 were obtained using

Tax4Fun2_ReferenceData_v2 and Ref99NR in *database_mode* with copy number normalization enabled. Only Kegg Ontologies (KO) shared between the approaches were used for functional pathway estimation downstream. Shared KOs were searched in the PICRUSt2 generated *pred_metagenome_contrib.tsv* file and corresponding ASVs (in counts) were extracted, these absolute counts were then used to draw functional abundance plots. KEGG Pathway (<https://www.genome.jp/kegg/pathway.html>) was used to identify the KO's involved in carbon, nitrogen and sulphur metabolic pathways.

Statistical analysis

The significance level for statistical analyses was 0.05. Observed richness (α -diversity) and Pielou's evenness indices of coral skeleton and tissue were computed on unrarefied ASV tables. The influence of pH and O₂ gradients on the microbial community's β -diversity were computed on centre log-transformed Euclidean distance matrices of the ASV tables through permutational analysis of variance (PERMANOVA). The influence of pH and O₂ gradients on specific bacteria were assessed on the 200 most abundant ASVs via CCA (Ter Braak 1987), using Hellinger transformed, rarefied ASV tables (10,000 sequences per sample). CCA is a multivariate method that can disentangle patterns or changes in biological communities and identify potential associations between explanatory variables and ASVs (based on their coordinates' similarity on the CCA plots). CCA analysis was performed on the top 200 most abundant ASVs of each dataset, both to reduce its dimension and because rare species may have an unduly large influence on these types of analysis (Legendre and Gallagher 2001). Downstream statistical analyses were conducted using RStudio version 1.2.5033 and packages *ampvis2* (Andersen et al. 2018), *decontam* (Davis et al. 2018), *ggfortify* (Tang et al. 2016), *ggplot2* (Wickham 2011), *ggvegan* (Simpson 2015), *microbiome* (Lahti 2012), *phyloseq* (McMurdie and Holmes 2013), *rgr* (Garrett 2013) and *vegan* (Oksanen et al. 2017).

Results And Discussion

Stratification of endolithic communities in the coral skeleton

The α -diversity (observed diversity and Pielou's evenness) of the bacterial community increased from shallower to deeper skeletal layers (Fig. 2a-d). In the vertical cross-section of the coral skeleton, the observed bacterial diversity in *Po. lutea* and *Pa. australensis* increased gradually (Fig. 2a and 2b), while Pielou's evenness of the bacterial communities of both coral species first increased steeply between 0 and 8 mm (more pronounced in *Po. lutea* than in *Pa. australensis*) and then slightly between 8 and 20 mm (Fig. 2c and 2d). Principal component analysis (PCA) biplots of the β -diversity of the bacterial communities of *Po. lutea* and *Pa. australensis* show a progressive increase in dispersion of samples moving from shallower to deeper skeletal layers (Fig. 2e and 2f). In biological terms, these α - and β diversity metrics show that the endolithic bacterial communities of both coral species were more diverse and evenly distributed in deeper skeletal layers. The data suggest that the deep skeleton may offer a suitable environment to a wider range of bacteria, a similar pattern to that observed in stromatolites, where deeper and more physicochemically stable layers have been shown to harbour a broader bacterial diversity (Toneatti et al. 2017).

Hyperspectral reflectance imaging on vertical cross-sections of *Po. lutea* and *Pa. australensis* coral colonies revealed a diversity of chlorophylls (Chl) and bacteriochlorophylls (Bchl) sustaining both oxygenic and anoxygenic photosynthesis in the coral skeleton (Fig. 3; Supp. Figure 3). Our 16S and 18S rRNA gene datasets contained 375 and 203 unique ASVs attributable to bacterial and eukaryotic phototrophs (Supp. Table 1), respectively, supporting the hyperspectral imaging results. However, the distribution of photosynthetic pigments was different in the two coral species, where pigments showed a clear stratification in *Po. lutea* and a more patchy distribution in *Pa. australensis* (Fig. 3; Supp. Figure 3). This suggests that the skeletal architecture dimmed the light environment differently (Marcelino et al. 2013) and consequently affected the distribution of the photosynthetic pigments. These results support previous findings (Fordyce et al. 2021) where chlorophyll concentrations correlated with the skeletal morphology characteristics of each coral species. Chl *a* was more abundant in shallower layers in the proximity of the tissue than in deep skeletal layers (> 2 cm from the coral tissue; Fig. 3; Supp. Figure 3). Although signals of Chl *a* in the deep skeleton indicate the presence of oxygenic phototrophs such as *Cyanobacteria* and endolithic algae, our data cannot confirm whether these chlorophyll pigments were photosynthetically active or rather remaining pigments from older microbial populations.

In both coral species, the phyla *Proteobacteria* (Orders: *Rhizobiales*, *Cellvibrionales*, *Rhodobacterales* and *Rhodospirillales*) and *Chlorobi* (Order: *Chlorobiales*) dominated the phototrophic community (Supp. Table 1). Signals of Bchl *a* and *c* were present across

the vertical cross-sections of both *Po. lutea* and *Pa. australensis* skeletons (Fig. 3; Supp. Figure 3), albeit with stronger Bchl *a* signals in deeper skeletal layers of *Po. lutea*. These results suggest that members of the *Chlorobi* that have both Bchl *a* and *c* may colonize the whole *Po. lutea* skeleton, while members of the *Proteobacteria* that only have Bchl *a* were mainly confined to deeper skeletal layers. In line with the hyperspectral imaging data, the number of phototrophic bacterial ASVs increased with increasing depth in the coral skeleton (Supp. Figure 4), an environment generally regarded as more stable in comparison to shallower skeletal layers (Magnusson et al. 2007; Kühl et al. 2008). The lower biodiversity of shallower phototrophic communities in the coral skeleton could also be explained by coral host-bacterial interactions. For instance, it has been proposed that members of the *Chlorobi* could interact with the coral host and remove H₂S from shallower skeletal layers (Cai et al. 2017). H₂S is toxic for corals but necessary for chemolithotrophic sulphur oxidising bacteria (Pokorna and Zabranska 2015).

Physicochemical gradients in the skeleton

Chemical imaging shows that the coral skeleton microenvironment was characterized by O₂ and pH gradients (Fig. 4; Supp. Figure 5; Supp. Table 2). Chemical imaging provides fine-scale measurements in structurally complex systems (Santner et al. 2015) but our experimental setup had limitations. For instance, in our experiments the coral cross-sections were illuminated through the transparent planar optodes and the skeleton cross-section borders were sealed using black plasticine. These procedures prevented us to take into account the natural vertical light gradient that penetrates the skeleton and measuring the influence of the tissue layer on the O₂ and pH gradients.

Hyperspectral imaging data showed apparent absorption in the ranges 600–640 nm (phycobiliproteins), 660–680 nm (Chl *a*) and 700–740 nm (far-red shifted Chl *a*, Chl *d* and/or Chl *f*) in the skeletons of both coral species (Fig. 3; Supp. Figure 3; Robertson et al. 2001; Antonaru et al. 2020) that suggest the presence of oxygenic phototrophs and align with the detection of *Chlorophyta*, *Stramenopiles*, and *Rhodophyta* in our 18S rRNA data (Supp. Table 1). Our results also support previous studies showing that skeletal oxygenic phototrophs absorb far-red wavelengths (Magnusson et al. 2007; Kühl et al. 2008; Fordyce et al. 2021; Ricci et al. 2021) and through their metabolism influence the physicochemical environment of the skeleton (Fig. 4; Shashar and Stambler 1992; Kühl et al. 2008).

The cross-section of all coral skeleton samples exhibited some O₂ production by endoliths when illuminated, but the bulk skeletal environment remained anoxic (Fig. 4). Peak concentrations of dissolved O₂ occurred in shallower skeletal layers of *Po. lutea* (0–8 mm) but was found deeper in the skeleton of *Pa. australensis* (4–16mm; Fig. 4; Supp. Table 2). Our results confirm that O₂ production can be observed inside the coral skeleton (Bellamy and RiSK 1982; Shashar and Stambler 1992; Kühl et al. 2008) and show that different coral species are characterized by different O₂ gradients. Oxygenic phototrophs induce oxygenation and alkalization of the skeleton through photosynthesis, but this is not the only functional group influencing the O₂ and pH gradients through their metabolism. For instance, denitrifiers and sulphate reducers induce alkalization (Rust et al. 2000; Tran et al. 2021) and microbial respiration limits build-up of O₂ and an increase in pH (Berggren et al. 2012). In *Po. lutea*, O₂ and pH gradient peaks were in the same skeletal layers under irradiance levels of ~ 8 and ~ 21 μmol photons m⁻² s⁻¹, while the two gradients seemed disconnected in *Pa. australensis* (Fig. 4). In *Po. lutea* the pH peaks were between 4 and 16 mm from the colony surface (Fig. 4; Supp. Table 2), while the pH in *Pa. australensis* increased consistently from shallower to deeper skeletal layers (Fig. 4; Supp. Table 2). In the natural environment, the physicochemical properties of each coral species and colony thus result from more complex interactions between coral tissue thickness, skeletal architecture, autotrophic and heterotrophic metabolisms.

The inferred functional profile of the endolithic community

Using computational predictions of function from the 16S rRNA gene, we predicted that the bacterial community was involved in carbon, nitrogen and sulphur metabolic pathways (Supp. Fig. 6) and potentially could provide pyruvate, acetyl-CoA, fixed nitrogen and sulphur to the coral holobiont. These metabolic pathways are tightly interwoven and some of their reactions are O₂ and pH-sensitive (Šimek et al. 2002; Pratscher et al. 2011), therefore their spatial-temporal rates are likely to be affected by the physicochemical gradients of the skeletal environment.

Skeletons of *Po. lutea* and *Pa. australensis* showed high numbers of ASVs potentially involved in the transformation of inorganic and organic carbon compounds via pathways like 3-hydroxypropionate bicycle, acetate kinase-PO₄³⁻ acetyltransferase, Wood-

Ljungdahl and reverse Krebs cycle throughout the coral skeleton cross-sections (Supp. Fig. 6). These pathways are thought to encompass various physicochemical requirements (e.g. presence/absence of O₂, low pH, high temperature) and trophic strategies (e.g., chemoautotrophs, chemoheterotrophs) (Ingram-Smith et al. 2006; Tabita 2009; Bar-Even et al. 2012; Weiss et al. 2016). Microbes use these pathways to convert inorganic and organic carbon compounds to energy and molecules such as acetyl-CoA and acetate (Quayle 1972; Ingram-Smith et al. 2006; Tabita 2009; Pratscher et al. 2011; Bar-Even et al. 2012; Weiss et al. 2016). For example, the 3-hydroxypropionate bicycle is used by bacteria in aerobic environments to autotrophically fix CO₂ and produce pyruvate through a series of reactions (Hügler and Fuchs 2005). This bicycle was mainly predicted by our data in the deep skeleton (Supp. Fig. 6), an environment where we measured low O₂ but a high abundance of *Chloroflexi* (Supp. Table 1), which are the only bacteria known to use this bicycle (Bar-Even et al. 2012). By way of another example, bacteria use the reverse Krebs cycle to fix carbon and, through a series of reactions, synthesise molecules such as acetyl-CoA and pyruvate (Tang and Blankenship 2010; Bar-Even et al. 2012). This cycle is thought to be restricted to anaerobic environments but studies suggest that bacteria can also operate it in presence of O₂ (Bar-Even et al. 2012) and accordingly our data predicted the presence of this cycle both in aerobic and anaerobic skeletal areas (Supp. Fig. 6).

We predicted the involvement of endolithic bacteria in six pathways associated with nitrogen metabolism (Supp. Fig. 6). Our data show that *Po. lutea* had more ASVs associated with diazotrophic bacteria than *Pa. australensis* and their abundance was higher in the deeper and less oxygenated skeleton (Supp. Fig. 6). Assimilatory and dissimilatory NO₃⁻ reduction were among the two most abundant predicted nitrogen pathways in both coral species (Supp. Fig. 6). Through the assimilatory pathway, NO₃⁻ is reduced to NH₄⁺ and incorporated as organic nitrogen (Sias et al. 1980). In the dissimilatory pathway, NO₃⁻ is used as an electron acceptor and reduced through a series of steps to N₂ (Sias et al. 1980), thus allowing bacterial growth in environments that lack O₂ like the coral skeleton. Many bacterial ASVs could be responsible for reductive pathways (e.g. dissimilatory and assimilatory NO₃⁻ reduction, anammox and denitrification), while there were few ASVs that could be responsible for nitrification, whose end product is NO₃⁻ (Supp. Fig. 6). Previous studies that measured NO₃⁻ in the skeleton found contrasting results on the concentration of this form of inorganic nitrogen (Risk and Muller 1983; Ferrer and Szmant 1988). One study (Risk and Muller 1983) suggested that skeletons characterized by nearly anoxic conditions, like those measured in our study (Fig. 4), promote reductive pathways, while skeletons that show higher O₂ concentrations facilitate oxidative pathways. Our results are in line with this concept. However, since we found few ASVs that could be responsible for nitrification (Supp. Fig. 6) our data leave an open question regarding the origin of NO₃⁻ necessary to feed the reductive pathways occurring within the skeleton. Although nitrogen is a growth-limiting nutrient (Kuypers et al. 2018), our data show that endolithic bacteria had the potential to contribute to the nitrogen budget of the coral holobiont with essential compounds like NH₄⁺ (Supp. Fig. 6).

Metabolic pathways associated with sulphur metabolism were among the most predicted in deep skeletal layers of both coral species (Supp. Fig. 6). Sulphate reduction is a predominant pathway largely restricted to anaerobic environments like the deep skeleton (Wasmund et al. 2017; Fig. 4; Supp. Fig. 6). However, we also predicted the presence of this pathway in shallower skeletal layers (Supp. Fig. 6) that, when illuminated, showed higher O₂ build-up (Fig. 4). These results suggest that part of the skeleton could be characterized by temporal compartmentalization of metabolic functions (e.g. photosynthesis in daylight and sulphate reduction in darkness). The coral skeleton is an environment enriched in sulphur (Clode and Marshall 2003; Cuif et al. 2003) and accordingly, metabolic pathways involving the processing of sulphur compounds of intermediate oxidation states were predicted in abundance across the whole coral skeleton cross-section (Supp. Fig. 6). The carbon, nitrogen and sulphur metabolic pathways predicted through our data analysis support the hypothesis that endolithic bacteria can be considered major nutrient recyclers (Fine and Loya 2002; Sangsawang et al. 2017; Moynihan et al. 2021) and, by showing how the abundance of these pathways changes across the skeleton depth gradient, we provide new insight into the spatial distribution of the coral skeleton biogeochemical cycle.

The ecological microniches of the coral skeleton

The skeletal microniches were characterized by the presence of dynamic O₂ and pH gradients (Fig. 4; Supp. Fig. 5; Supp. Table 2) and harboured microbial communities that varied in their composition with depth in the skeleton (Fig. 5). PERMANOVA showed that dissolved O₂ concentrations and pH distributions measured in *Po. lutea* and *Pa. australensis* skeletons correlated with the β-

diversity of their endolithic bacterial communities (Supp. Table 3). Considering these results, we investigated whether the abundance of specific bacterial taxa showed associations with the physicochemical properties of the skeleton, and we found that bacterial ASVs correlated with dissolved O₂ and pH gradients in the CCA biplots in both coral species (Fig. 6; Supp. Fig. 7 and 8; Supp. Table 4 and 5). These results suggest that the overall bacterial community composition and the presence and abundance of certain endoliths are influenced by the physicochemical environment of the skeleton.

The spatial heterogeneity of the physicochemical environment differed between the two coral species and was possibly influenced by their skeletal architecture (Fig. 5). In *Po. lutea*, O₂ and pH peaks were constrained to skeletal layers with abundant endolithic algae (Fig. 4a and 4b; Fig. 5a; Supp. Table 2), suggesting that the more homogeneous and denser skeleton of this species limited gas and solute diffusion. In *Pa. australensis*, peaks in dissolved O₂ concentration corresponded to skeletal areas with abundant endolithic algae and the shape of the O₂ gradients was possibly determined by the more perforated skeletal architecture of this species (Fig. 4c and 4d; Fig. 5b; Supp. Table 2). Interestingly, the pH of *Pa. australensis* increased deeper in the skeleton (Fig. 4c and 4d; Fig. 5b), where we found ASVs belonging to *Bacillus* and *Spirochaetaceae* (Fig. 5d) that may increase the skeletal matrix pH through denitrification (Wei et al. 2015).

Increased O₂ and pH values in *Po. lutea* skeletons correlated with ASVs belonging to the nitrogen fixing genera *Spirochaeta* and *Tistlia* (Fig. 6a) and in *Pa. australensis* with *Alteromonas* and *Pseudoalteromonas* ASVs that are thought to take part in nitrogen cycling and antibacterial activity (Fig. 6b; Shnit-Orland et al. 2012; Ceh et al. 2013a; Ceh et al. 2013b). In *Po. lutea*, ASVs of the obligate anaerobes *Chlorobi* correlated with higher O₂ values, suggesting that these bacteria were likely to be found in skeletal layers characterized by higher but still hypoxic O₂ concentrations (Fig. 6a). The occurrence of these presumed obligate anaerobes in presence of O₂ is unexpected, but *Chlorobi* have been previously reported in coral tissue (Cai et al. 2017) and skeletons with abundant endolithic algae (Marcelino and Verbruggen 2016). It is possible that in our study the presence of *Chlorobi* in hypoxic skeletal layers resulted from their interactions with other holobiont members rather than being a direct response to the O₂ gradients. For instance, it has been proposed that these bacteria could remove toxic H₂S generated by sulphate-reducing bacteria (Cai et al. 2017). Accordingly, we found that predicted sox system pathways (Fig. 5a; Supp. Fig. 6), through which bacteria like *Chlorobi* oxidize H₂S (Friedrich et al. 2001), were present in shallower layers of *Po. lutea* skeletons. In *Pa. australensis*, ASVs of another presumed strictly anaerobic bacterium, *Paramaledivibacter*, correlated with elevated O₂ concentrations and higher pH values (Fig. 6b). These bacteria have also been found in the tissue of other coral species (Santoro et al. 2021; Ricci et al. 2022), but identification of their physiological requirements in the coral holobiont awaits further investigation.

Conclusion

The skeletal microenvironments of *Po. lutea* and *Pa. australensis* were characterized by microniches that are shaped by the metabolism of the endolithic microbiome, and these microniches, in turn, harboured divergent microbial communities likely adapted to the physicochemical gradients of the skeleton. The unique skeletal architecture of each coral species influenced the light available to phototrophic endoliths (Enríquez et al. 2005; Marcelino et al. 2013), which through their metabolisms determine the physicochemical environment and ultimately the microbiome. Superimposed onto this, molecules diffuse differently in the compact and dense skeletons of *Po. lutea* compared to the heterogeneous and perforated skeletons of *Pa. australensis* (Wu et al. 2009) and contribute to shaping the physicochemical environment. We also note that other important factors were not measured in this study, such as the availability of electron donors and acceptors, take part in structuring the microbial community. The coral skeleton can be compared to other microbial systems. For instance, microbial metabolism induces abrupt physicochemical changes in stromatolites, with observations reporting shifts from high irradiance and O₂ presence in outer layers to predominance of infrared light, absence of O₂ and high H₂S in the inner layers (Toneatti et al. 2017). These conditions, in stromatolites like in the coral skeleton, force stratification and metabolic adaptations of the microbial community.

With this study, we have started to link the physicochemical, microbial and functional landscapes of the coral skeleton and inferred the involvement of the skeletal microbiome in the holobiont metabolic processes. A more comprehensive picture of the mechanisms governing this intriguing system will be gained by mapping other physicochemical gradients like NH₃ or H₂S and by characterizing the spatial heterogeneity of biochemical activity taking place across the coral skeleton and the holobiont more broadly.

Declarations

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Author's contribution

F.R., L.L.B., M.K. and H.V. contributed to the conceptual development of the manuscript. F.R. and M.K. conducted the experiments. F.R., K.T., M.M., E.H.J.C., M.K. and H.V. conducted the data analysis. F.R. wrote the first draft of the manuscript and all authors contributed to the final edited version of the manuscript.

Data availability

High quality figures, supplementary figures and tables, analysis workflow and datasets are available at https://melbourne.figshare.com/projects/Linking_the_microbial_and_physicochemical_landscapes_of_the_coral_skeleton/130694. Sequence data determined in this study are available at NCBI under SRA Accession PRJNA753944 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA753944>).

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Supplementary Material

Supplementary Figures and Tables are not available with this version.

Figures

Figure 1

Vignette of the study experimental design. The coral skeletons were first cut into two halves (side A and B). Side A was used to map the O₂ and pH concentration over the skeleton cross-section through chemical imaging and photopigment distribution through hyperspectral reflectance imaging. Side B was used to for collecting five microsamples of 64 mm³ that were used to characterize the bacterial and eukaryotic communities through 16S and 18S rRNA genes metabarcoding and infer the metabolism of the bacterial community through functional prediction software. The results of these methodologies were integrated and properties of the physicochemical environment were linked to the structure and functions of the microbial community. Diagrams coloured in red show the methodologies used to map the physicochemical environment, green to characterise the microbial community and blue to infer bacterial functions.

Figure 2

Boxplots show α -diversity indexes, specifically observed diversity (a-b) and Pielou's evenness (c-d) of the bacterial communities of each skeletal layer of *Po. lutea* and *Pa. australensis* samples. PCA biplots show β -diversity of skeletal layers of *Po. lutea* (e) and *Pa. australensis* (f) bacterial communities. Data representing each skeletal layer are colour coded.

Figure 3

Hyperspectral reflectance imaging of *Po. lutea* FRH48 (a) and *Pa. australensis* FRH54 (b) skeletons showing their cross-section and spatial distribution of zones with similar reflectance properties. The graphs show the reflectance of each colour coded skeletal zone indicating apparent chlorophyll pigments composition. Specifically, absorption in the regions 620-640 nm suggest presence of phycobiliproteins, absorption in the 660-680 nm range suggests chlorophyll a, absorption in the 700-740 nm region suggests presence of chlorophyll d, f, or far-red chlorophyll a, absorption in the 740-760 nm region suggests presence of bchl c, and absorption in 785-815 and 835-855 nm region suggest presence of bchl a. Scalebars next to the skeletons cross-section are 1 cm. To inspect hyperspectral reflectance imaging data of each coral skeleton analysed in this study please see Supp. Fig. 3.

Figure 4

Cross-sections of skeletons, closeup on the skeletal architecture and chemical imaging of O₂ and pH gradients during homogeneous exposure of the cut surface of the sample *Po. lutea* FRH48 to incident photon irradiance (400-700 nm) of ~21 μmol photons m⁻² s⁻¹ (a) and of the sample *Pa. australensis* FRH52 to incident photon irradiance (400-700 nm) of ~50 μmol photons m⁻² s⁻¹ (c). Graphs of O₂ and pH (panels a and c) refer to the area underneath the dotted line in the chemical imaging figures. Boxplots show O₂ and pH values measured through chemical imaging in the skeleton of each *Po. lutea* (b) and *Pa. australensis* (d) sample during homogeneous exposure of the cut surface to incident photon irradiance (400-700 nm) of ~8, ~21 and ~50 μmol photons m⁻² s⁻¹. Scale bars of skeletons cross-sections and chemical imaging are 1cm. Skeletal architecture scale bars are 1mm.

Figure 5

Links between microbial community composition, physicochemical gradients and inferred functions of each skeletal layer in *Po. lutea* FRH49 (a) and *Pa. australensis* FRH54 (b). The panels show from left to right the bacterial and eukaryotic microbiome, the respective skeletal section, the pH and O₂ gradients measured during homogeneous exposure of the cut surface to incident photon irradiance (400-700 nm) of ~21 μmol photons m⁻² s⁻¹, and sulphur, nitrogen and carbon inferred functional profiles. The heatmaps show the relative abundance of the 30 most frequent bacterial ASVs in each skeletal layer across every *Po. lutea* (c) and *Pa. australensis* (d) sample. By applying black plasticine along the coral perimeters, exchanges of gas and fluids between the aquaria, the coral tissue and the skeleton cut surface were impaired, thus the physicochemical gradients reported in this study (O₂ and pH) are free from the coral tissue interference. In panels a and b, the microbial groups that include oxygenic phototrophs are indicated with *.

Figure 6

CCA biplot representing *Po. lutea* (a) and *Pa. australensis* (b) bacterial communities structure according to the explanatory quantitative variables O₂ and pH (arrows) measured during homogeneous exposure of the cut surface to incident photon irradiance (400-700 nm) of ~50 μmol photons m⁻² s⁻¹ (a) and ~21 μmol photons m⁻² s⁻¹ (b). For CCA biplots showing all the bacterial taxa refer to Supplementary Figures 7 and 8, and Supplementary Tables 5 and 6.

Supplementary Files

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- [SupplementaryLegends.docx](#)