Metatranscriptomics sheds light on "who is doing what" in the coral *Porites lutea*

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Abstract

Global decline of coral reefs due to climate change calls for effective, nature-based strategies to protect these crucial ecosystems. Developing such strategies requires a thorough understanding of the complex roles and interactions of key inhabitants within coral holobionts. Using a metatranscriptomics approach, we investigated the transcriptionally active microbiome of the healthy stony coral *Porites lutea*, focusing on the coral tissue, the green endolithic algal layer (*Ostreobiu*m layer), and the deeper coral skeleton. We show distinct, metabolically active communities within these compartments and highlight substantial metabolic redundancy across carbon, nitrogen, and sulphur pathways. Our study provides the first transcriptomic evidence of the ability of *Ostreobium* to transfer fixed carbon to other members of the holobiont and the coral host. Additionally, we identified critical roles of diverse coral holobiont members in nutrient cycling and maintaining homeostasis through scavenging of reactive oxygen and nitrogen species. This study provides novel molecular-level understanding of the functional roles played by diverse coral holobiont members in their respective compartments and underscores that corals harbour several distinct microbiomes with a wide array of functions.

Introduction

Coral reefs owe their success to the symbiosis between reef-building, stony corals (Scleractinia) (*1–3*) and a large diversity of closely associated bacteria, archaea, viruses, and microeukaryotes, collectively termed the coral holobiont (*3–6*). These symbionts *sensu lato* encompass organisms spanning the full spectrum of host-microbe interactions, from mutualistic partners that enhance coral fitness to opportunistic pathogens, as well as commensals and other microorganisms whose ecological roles may shift along this continuum depending on environmental conditions. While the most well-characterized symbiosis in corals involves photosynthetic, endosymbiotic microalgae in the family Symbiodiniaceae residing in the gastrodermal tissues of the animal host (*7–9*), a compendium of prokaryotes associated with corals has identified 39 bacterial and 2 archaeal phyla (*10*). This diverse microbial assemblage is not only distinct between different compartments within the coral (*11*), but fine-scale mapping of the coral skeleton has revealed a stratified community across its depth (*12–14*). In recent years, other microeukaryotes besides Symbiodiniaceae, including endolithic algae of the genus *Ostreobium*, fungi, and apicomplexans have also garnered attention (*15*, *16*).

The diversity and composition of coral holobiont members have been investigated through metabarcoding of ribosomal marker genes (16S rRNA, 18S rRNA, ITS2) (*17–19*) (*20*, *21*),providing invaluable insights into the diverse and structured symbiont composition *sensu lato* within coral compartments (*12*, *13*, *22*, *23*). Combined culture-based and molecular approaches have expanded our understanding of the functions of coral holobiont members in maintaining coral health and resilience (*24–28*). Symbiodiniaceae, in particular, play a crucial role by supplying corals with photosynthates, meeting a significant portion of their energy demand (*29*). Coral-associated bacteria and archaea have been implicated in a myriad of functions essential for sustaining coral health and resilience, including stress alleviation through scavenging of reactive oxygen and nitrogen species (ROS and RNS), protection against pathogens and nutrient cycling (*24–26*, *28*, *30–32*). Coral skeleton-dwelling endolithic green algae of the genus *Ostreobium,* which form conspicuous green bands beneath the coral tissue, have also been suggested to support corals by providing photosynthates during coral bleaching (*33–35*), but we lack a detailed understanding of the metabolic interaction between the coral animal and *Ostreobium* (*15*).

Most of our present knowledge on the role of microbiota for coral function and health comes from amplicon and metagenomic sequencing studies, with potential functions inferred using correlative, bioinformatic analyses. Despite these advances and the extensive information now available on the gene-encoded functional potential of microbiome and microeukaryotic members through genomic and metagenomic studies, a significant knowledge gap persists

regarding actual microbial metabolic activity and its spatial organisation within the coral holobiont. Genomic data alone cannot predict the actual contributions of specific functions by symbionts *in situ*, as genes encoding these functions may be variably expressed or not expressed at all. Metatranscriptomics enables the analysis of the pool of actively expressed genes in an environmental sample, highlighting the ecophysiologically relevant processes occurring at the time of sampling (*36*). Therefore, metatranscriptomics can provide insights into which community members perform which functional roles.

Obtaining a molecular level understanding of the functional roles of holobiont members is crucial especially given the rapid decline of coral reefs worldwide due the adverse effects of climate change (*37*). Addressing this knowledge gap will also be beneficial for developing effective nature-based interventions to protect coral reefs, including improved assisted evolution strategies and development of coral probiotics (*2*, *38*). Both of these approaches rely on a comprehensive understanding of the roles played by diverse microbial symbionts in maintaining coral health.

In this study, we aimed to address the fundamental question of "who is doing what" in a healthy coral holobiont across different compartments. Using metatranscriptomics, we aimed to identify the active symbiont community of the coral *Porites lutea*, characterising their specific activities and where these are carried out in the coral, from the coral tissue to the endolithic algal layer (hereafter *Ostreobium*-layer) and the deeper coral skeleton. Our focus was on key nutrient cycling pathways, including carbon-fixation (C), photosynthesis (P), nitrogen (N) and sulphur (S) metabolism, sugar-export, and the scavenging of reactive oxygen and nitrogen species (ROS and RNS).

Methods:

Coral sampling and colony fragmentation Fragments from six healthy-looking, *Porites lutea* colonies, spaced at least 15 m apart, were collected at low tide (1-2 m depth) from the research zone of the Heron Island reef flat, southern Great Barrier Reef (23°44′S, 151°91′E), in October 2022. Four fragments (from colonies we numbered 1, 3, 4 and 5) were collected during daytime, while two (colonies 6 and 7) were collected five-six hours after sunset. All collected colonies exhibited a visually discernible *Ostreobium-layer* in the coral skeleton . 10-50 mm below the coral tissue layer (Fig. 1A). The fragments were collected using a sterile hammer and chisel and were placed in separate sterile zip-loc polyethylene bags in seawater and flash frozen in liquid nitrogen immediately upon collection. A 5-mm thick slice was cut from the frozen samples, perpendicular to the vertical growth axis, using a diamond saw with a continuous flow of sterile filtered (0.2 µm) seawater. Later, pliers were used to obtain fragments from three distinct layers, i.e., the coral tissue, the green *Ostreobium*-layer,

and the tan fraction of the skeleton below the green band (termed the skeleton layer). During this process, the material was kept frozen by repeated immersion in liquid nitrogen. Each layer was kept frozen at -80ºC, transported to the University of Melbourne on dry ice, and processed immediately upon arrival.

Total RNA extraction

For RNA extraction, samples of individual layers were ground to a fine powder in liquid nitrogen using pre-cooled mortars and pestles. Approximately 200 mg of powdered tissue samples and between 300-500 mg of powdered *Ostreobium-layer* and skeleton layer were used for RNA extraction with 1 mL of pre-cooled TRIzol reagent (Invitrogen, USA). Total RNA was extracted following the manufacturer's protocol with some modifications (**see supplementary file 1 for details**). Quantification of total RNA was conducted using the Qubit RNA HS Assay kit (Thermo Fisher Scientific, USA).

Metatranscriptome sequencing and data-preprocessing

We did not perform any ribosomal RNA depletion due to the dual aim of sequencing mRNA and rRNA from both eukaryotes and prokaryotes simultaneously, and profiling the transcriptionally active microbial and microeukaryotic community composition using 16S and 18S rRNA abundance (see below). Total RNA libraries were prepared for 36 samples (6 colonies x 3 layers x 2 replicates per layer) and sequenced on the Illumina NovaSeq platform by Azenta (Suzhou, China). Sequencing yielded approximately 23 million reads per sample with 2x150 bp reads.

Raw reads were quality checked and trimmed using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trimmomatic v0.39(*39*), respectively. Trimmomatic parameters were as follows: *TruSeq3-PE.fa:2:30:5 HEADCROP:5 SLIDINGWINDOW:4:20 MINLEN:50*. Quality-filtered reads were first used to identify and filter out rRNA reads using SortMeRNA v4.3.6(*40*) with Silva v138.1 LSU and SSU (*SILVA138.1 LSU and SSU NR99 database, last accessed Feb 10, 2023*)(*41*). Reads identified as rRNA were taxonomically classified using PhyloFlash v3.4(*42*) (with *almost-everything*), which rapidly reconstructs the SSU rRNAs (NTUs, taxonomic units) and taxonomically annotates them using Silva 138.1 NR99 SSU database. Reads identified as rRNA were removed from the raw-read set, and non-rRNA reads were mapped to the *Porites lutea* genome (accessed from www.reefgenomics.org) using bowtie2 v2.3.4.1(*43*) and samtools 1.17(*44*) to identify and remove host-related reads.

High-quality, non-rRNA and non-host reads were co-assembled using Trinity v2.15.1(*45*) with parameters *--min_contig_length 250 and --no_salmon*. Taxonomic annotation of assembled transcripts was obtained using the lowest common ancestor approach by

performing *blastx (46)* searches against the NR database (last accessed April 2023) with diamond v2.1.6.160(*47*), using an *e-value cutoff of 1e-5* and processed through MEGAN6 community edition v6.24.20, using the *megan-map-Feb2022-ue.db* and meganising the *daa* file using *daa2info* (*48*). This process was used to identify and remove any transcripts annotated as Scleractinia and other Eumetazoa. The remaining transcripts were quantified using RSEM (RNA-Seq by Expectation and Maximisation)(*49*), implemented in Trinity through the utility script *align_and_estimate_abundance.pl*. This approach generated both gene and isoform expression profiles. Isoform-level expression data was used for the analysis. Expression values per transcript were averaged across replicates for each layer (tissue, *Ostreobium*-layer and skeleton).

Community composition analysis

Taxonomic classification of NTUs assembled from rRNA reads was conducted to profile the abundance of transcriptionally active community members. NTUs classified as “Chloroplast” or “Mitochondria” were removed. Abundance values were converted to relative abundance for compositional analysis using barplots and log-transformed for diversity analysis using PCoA. These analyses were performed using R packages phyloseq v1.42.0(*50*), metagMisc v0.5.0 (https://github.com/vmikk/metagMisc) and ggplot2 v34.3 (*51*). We used the quantified and taxonomically annotated transcripts assembled from mRNA reads to profile the community composition encoding for different functional guilds based on the metatranscriptome data and compared them to rRNA based analysis using the same approach.

Functional analysis

To ascertain functional roles, we first filtered transcripts using Trimmed Mean of the M (TMM) values of 0.1 and presence in at least 2 samples to identify truly expressed transcripts. This approach filters out i) any transcripts from potential residual DNA or very low expressed transcripts, ii) transcripts originating from spurious mapping of reads. Transcripts deemed truly expressed were then grouped in a compartment-specific manner to obtain tissue, *Ostreobium*-layer and skeleton-layer specific metatranscriptome. These compartment specific transcripts were processed through Trinotate v4.0.0(*52*) and Transdecoder v4 (https://github.com/TransDecoder/TransDecoder) to identify the longest open reading frames and predict the proteins from transcripts ≥50 amino acids. Predicted proteins were functionally annotated using a combination of EggNOG mapper v2.1.12(*53*) with database eggnogDB v5.0.2(*54*) with *e-value cutoff 1e-5*, ghostkoala (*55*) with database *c_family_euk+genus_prok+viruses* parameters and Interproscan v5.63-95.0(*56*) with *e value*

cutoff 1e-5 to obtain a comprehensive understanding of functional orthologous groups (EggNOG and COG), KEGG pathways, including Kegg Ontology, KOs and protein families (pfam). Metabolic markers for carbon-fixation (C), photosynthesis (P), nitrogen (N) and sulfur (S) metabolism were identified from a previous study (*57*). Additionally, we focussed on genes involved in sugar export and scavenging of ROS and RNS. Expression profiles of transcripts functionally annotated with marker KOs or pfams were plotted using heatmaps using ComplexHeatmap(*58*) in R.

Results and Discussion

Corals harbor compartment-specific active symbiont community

One of our primary objectives was to profile the transcriptionally active community within the coral holobiont and compare these findings with the community composition obtained from rRNA analysis. To achieve this, we examined the total and active community composition across the coral tissue, the endolithic *Ostreobium-layer*, and the underlying coral skeleton, and compared it with their rRNA based community composition (**Fig 1A)**. For total community composition, we taxonomically analysed 28,717 rRNA NTUs (6,010 from tissue layer, 15,942 from *Ostreobium* layer, 26,712 from skeleton layer) belonging to Archaea (2.92% - 840 sequences), Eukaryota (12.68% - 3,644 sequences), and Bacteria (84.38% - 24,233 sequences) (**Fig 1B**). This observation offers essential insights into the diversity and composition of the coral holobiont. Unlike traditional amplification-based metabarcoding analyses, which are often biased (*59*) and fail to provide accurate quantitative distinctions between prokaryotes and eukaryotes, using rRNAs assembled from total RNA delivers a more precise and comprehensive overview of the community composition. Eukaryotes (Amorphea, SAR, Archaeplastida) were the dominant groups in the coral tissue (**Supplementary Fig S1A**) and the *Ostreobium layer* (**Supplementary Fig S1B**), while the deeper skeleton was dominated by Bacteria (Proteobacteria, Planctomyceota, Desulfobacteria, and Cyanobacteria) (**Supplementary Fig S1C**). Our study shows that bacterial diversity is highest in the skeleton, where the majority of bacterial NTU sequences were assembled, confirming previous reports of greater bacterial richness in the skeleton compared to tissue compartments (*13*, *19*).

The active community composition was profiled using a curated set of 371,193 taxonomically annotated transcripts out of a total of 780,431 assembled. After excluding 111,367 transcripts belonging to Scleractinia, Chordata, and Eumetazoa, 259,826 (47.56%) transcripts remained. These transcripts represent Eukaryotes (52.48% - 136,366), Bacteria (44.99% - 116,900), Archaea (1.97% - 5,131), and viruses (0.54% - 1,429) (**Fig 1B**) and

revealed a high relative abundance of alveolates, the eukaryotic group containing Apicomplexa and dinoflagellates, including Symbiodiniaceae in coral tissue (**Supplementary Fig S2A)**. Green algae, Streptophyta and Chlorophyta, dominated the *Ostreobium layer* and deeper skeleton. (**Supplementary Fig S2B and C**). The high relative abundance of dinoflagellate (Symbiodiniaceae) expressed transcripts in coral tissue is unsurprising, as they are the most dominant microeukaryotes in the coral tissue, meeting most of the coral's energy demands (*60*). However, we identified dinoflagellate and Apicomplexa transcripts in all compartments. Apicomplexans have been found in healthy coral colonies across the geographic distribution and taxonomic diversity of corals, but their role is yet to be characterized (*16*, *61*). Bacteria and Fungi were present in all compartments, with bacteria as dominant members of the skeletal microbiome (**Supplementary Figure S2C)**. Proteobacteria, Cyanobacteria and Actinobacteria were the dominant bacterial phyla in all coral compartments. Members of the phylum Proteobacteria are some of the most widespread coral microbiome members (*10*, *17*, *62*). Cyanobacteria have also previously been identified as the dominant prokaryote group in the *Ostreobium layer* and deeper skeleton of *P. lutea* from the Red Sea (*63*). Obtaining annotation for less than 50% of the total assembled transcripts highlights the immense diversity of uncharacterised symbionts associated with *P. lutea,* despite it being one of the best investigated coral species with several metagenomic and physiological studies (*24*, *25*, *64*, *65*). These findings align with earlier studies using the metatranscriptomics approach to profile transcriptionally active microbiomes in other ecologically diverse systems, including soil (*66–68*), sponge (*69*) and ocean microbiomes (*57*, *70*).

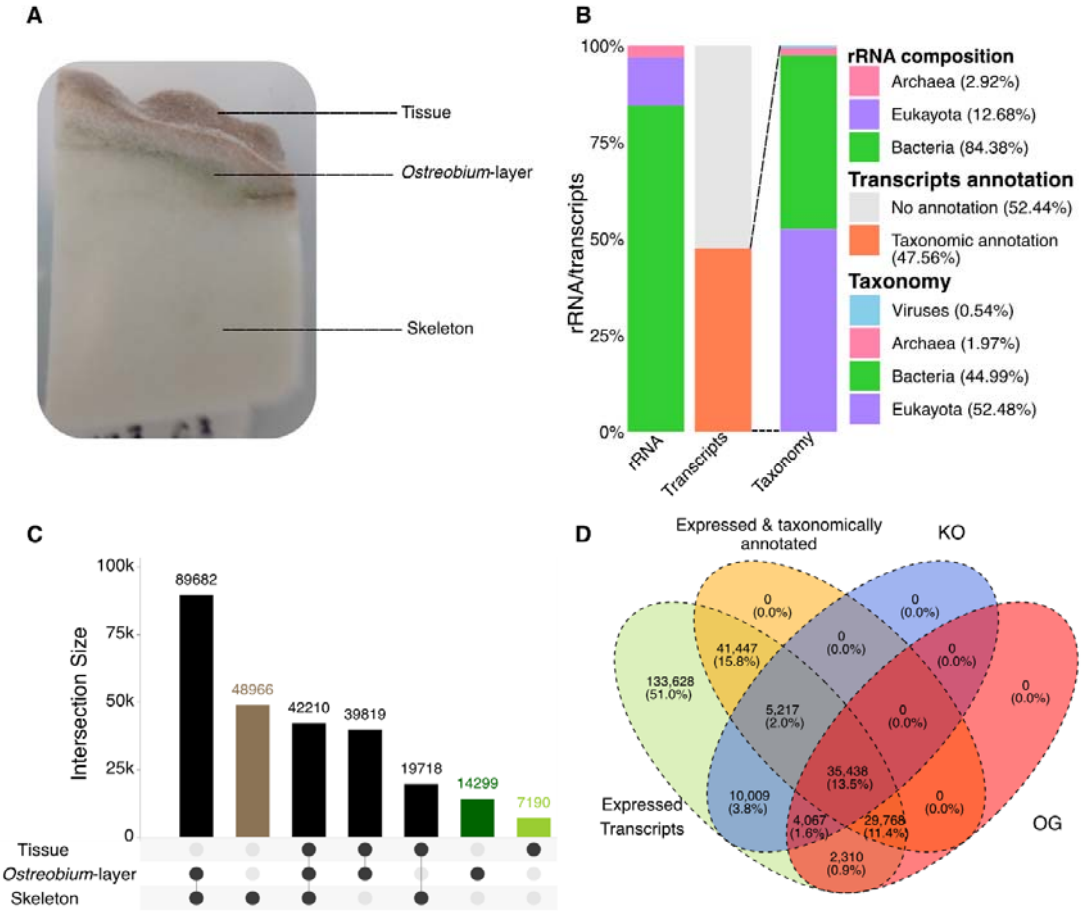

Fig. 1. Taxonomic and functional overview based on rRNA and transcriptome data A) illustration of a coral fragment depicting the three compartments: Tissue, Ostreobium-layer and Skeleton. B) Bar plots showing overall community composition based on rRNA and transcriptome data. C) UpSet plot illustrating shared and unique transcripts across different coral compartments. D) Venn diagram showing counts and percentage of transcripts which are deemed expressed, expressed and taxonomically annotated, annotated at KO and at OG levels.

Functional annotation of transcripts remains a bottleneck

Analysis of active metabolic pathways in a healthy coral holobiont revealed 261,884 truly expressed transcripts through TMM filtering. These transcripts were distributed across the three investigated compartments, with the highest overlap observed between the *Ostreobium layer* and deeper skeleton (89,682 shared transcripts). Additionally, a substantial number of uniquely expressed transcripts were identified in the deeper skeleton-layer (48,966), while the coral tissue exhibited the lowest count of uniquely expressed transcripts (**Fig. 1C**). Recent metabarcoding and metagenomic studies have highlighted the skeleton as the most diverse compartment of corals (*13*, *19*, *63*). In line with these observations, our

coral skeleton samples revealed 178,580 uniquely predicted proteins from the expressed transcripts. However, only 30.64% (54,731) could be annotated using KO ids and 29.25% (52,252) using OGs (**Fig 1D**). The extraordinary uniqueness of skeletal proteins underscores the added value of sampling genomically underexplored environments, such as the coral skeleton. The low functional annotation observed in our dataset is consistent with a recent study on gene expression changes and community turnover of global oceans, which similarly found less than 25% of the transcripts assigned to KOs (*57*). Annotations based on COG revealed a notable proportion of proteins encoded by transcripts involved in signal transduction, intracellular trafficking, secretion, vesicular transport, post-translational modification, protein turnover, and chaperones (**Supplementary Figure S3)**. These findings suggest intricate, interconnected metabolic interactions within coral compartments, facilitating the exchange of nutrients and metabolites. Such processes are likely crucial for coral health and stability, especially in nutrient-poor waters, and provide evidence of the dynamic roles microbial communities play in maintaining coral homeostasis through their contributions to essential nutrient cycles within the coral holobiont. However, to fully elucidate these nutritions exchanges, further experimental validation is required to identify metabolites being exchanged and the potential underlying mechanisms.

Distinct photosymbionts drive carbon fixation in coral compartments

To reveal the functional roles of transcriptionally active holobiont members in healthy *P. lutea* coral, we identified key microbial processes, such as photosynthesis, carbon fixation, sugar export, and nitrogen and sulphur cycling. Our analysis of pathway-specific metabolic markers highlighted the taxonomic affiliations of transcripts and their expression profiles, shedding light on the active contributions of each microbial group to these essential processes. The genes with the highest number of transcripts included those encoding photosynthetic markers for Photosystem I (PsaA, 59 transcripts) and Photosystem II (PsbA, 67 transcripts) and for subunits of RuBisCO(ribulose-1,5-bisphosphate carboxylase/oxygenase, *rbc*L (79) and *rbc*S (18) genes), the key enzyme in carbon-fixation via the Calvin-Benson cycle (**Fig 2**). Notably, while chemotrophs can also have a transcriptionally active *rbc*L gene (*71*), our study did not taxonomically annotate any chemotrophs, so the expression of these genes primarily reflects activities of phototrophs. These genes were actively transcribed by Symbiodiniaceae, stramenopiles, Bryopsidales (including the endolithic green alga *Ostreobium*), Cyanobacteria, Gammaproteobacteria, and unclassified Viridiplantae and Chlorophyta in different coral compartments. Symbiodiniaceae were the dominant contributors in coral tissue, while Cyanobacteria were active across all coral compartments. In the *Ostreobium*-layer and deeper skeleton layer, *Ostreobium* and

other Ulvophyceae were the key contributors (**Supplementary data file 2**). The expression levels of *rbc*L and *rbc*S correlated with those of *psb*A and *psa*A, indicating that carbon fixation is primarily driven by chlorophototrophs rather than chemotrophs, which derive energy from chemical sources (*72–74*). We identified the expression of coxS, coxM, and coxL transcripts in microbes from coral skeletons, primarily affiliated with Cyanobacteria and Proteobacteria, indicating the presence of carbon monoxide dehydrogenase (CODH) activity. This activity enables CO oxidation to CO_2, supporting energy generation through chemosynthesis. These findings suggest that these microbes contribute to carbon cycling and primary productivity in nutrient-poor, low-light environments. Their metabolic flexibility highlights a potentially critical role in supporting coral reef ecosystems through alternative energy pathways. Additionally, the expression of acs and acsB in the skeleton layers indicates the involvement of bacteria in carbon fixation via the Wood–Ljungdahl pathway, converting CO_2 and CO into acetyl-CoA, a key intermediate in various metabolic processes (*63*, *75*). The expression patterns of these genes underscore the metabolic diversity within the Ostreobium and skeleton-associated microbial communities, reflecting adaptation to specific environmental conditions, such as low O_2 (*14*, *76*) and the availability of CO, although the latter warrants further experimental validation. The conversion of both inorganic and organic carbon into energy may contribute to nutrient cycling, promoting microbial persistence and growth, and ultimately supporting the overall health and productivity of the coral holobiont.

Besides *psa*A and *psb*A, we observed expression of other photosynthetic marker genes, including those for the photosynthetic reaction centre, *petC* (encoding the Rieske protein, a part of the cytochrome b6f complex that connects Photosystem II to Photosystem I) and *petE* (encoding plastocyanin, which transfers electron between the cytochrome b6f complex and Photosystem II), across all three holobiont compartments. This indicates the presence of oxygenic phototrophs throughout the different layers of corals. Notably, genes encoding for cyanobacteria-specific antenna proteins (*apcA, apcF, cpcA, cpeA* and *cpeT*), were highly expressed in the *Ostreobium-layer* and deeper skeleton compared to the tissue, suggesting a higher prevalence and activity of Cyanobacteria in these compartments (**Fig 2**). This finding aligns with the taxonomic profiling at the rRNA (Supplementary Fig S1) and metatranscriptome levels (Supplementary Fig S2) and corroborates earlier reports showing a prevalence of genes with eukaryotic and cyanobacterial origins within the skeletal layers of *P. lutea (63)*. These genes are involved in the Calvin-Benson cycle and collectively represent >50% of the relative contribution to the total carbon fixation genes in the skeletal layers of *P. lutea* (*63*). Additionally, the expression of *pufM,* a marker for anoxygenic photosynthesis that encodes a protein component of the bacterial photosynthetic reaction

centre, was restricted to the coral skeleton, indicating the presence of obligate anaerobes, such as green sulphur and purple non-sulfur bacteria, in line with hyperspectral imaging of pigment distributions over coral skeleton cross-sections in *P. lutea* (*14*).

The observed differential expression patterns in the coral compartments reflect adaptations to varying light conditions and provide new insights into the metabolic flexibility of coral-associated microbes. The higher expression of cyanobacterial antenna proteins in the Ostreobium-layer and deeper skeleton, where light is limited, suggests that Cyanobacteria in these regions utilise alternative chlorophyll pigments, such as chlorophyll d and f, to absorb far-red light that penetrates through the coral tissue. This adaptation enables efficient photosynthesis under low-light conditions, supporting the role of Cyanobacteria in energy generation in these deeper layers. These findings extend earlier reports of the use of far-red absorbing pigments by Cyanobacteria and Ostreobium, which thrive in endolithic habitats with limited light (*77–79*). In the Ostreobium layer, the green alga *Ostreobium* also employs similar strategies, utilising chlorophyll b and enriching its repertoire of light-harvesting complex proteins in both photosystems to adapt to low-light conditions (*15*). This includes changes such as the loss of photoprotection genes and duplication of the *Lhca1* gene *(80)*. In contrast, the tissue compartment, exposed to higher light levels, harbours Symbiodiniaceae and other phototrophs adapted to higher irradiance, utilising chlorophyll a and c (*81*). The expression of specific photosynthetic genes across these compartments underscores the sophisticated strategies employed by coral-associated phototrophs, including *Ostreobium*, to thrive in their respective microhabitats. These findings highlight the metabolic diversity within the holobiont and contribute to our understanding of coral resilience in fluctuating environmental conditions.

Not only Symbiodiniaceae provide fixed carbon to the coral holobiont

To identify members of the coral holobiont responsible for exporting photosynthetically fixed carbon compounds (photosynthates), thereby potentially contributing to coral energy demands, we focused on genes encoding for "sugars will eventually be exported transporters" (SWEET) proteins. SWEET proteins act as bidirectional uniporters/facilitators, enabling the diffusion of sugars across cell membranes along concentration gradients (*82*, *83*). We identified the expression of transcripts encoding SWEET proteins (pfams: PF04193 and PF03083) across all three compartments in the coral holobiont (**Fig 2**). Taxonomic annotation of these transcripts identified eukaryotes, including Symbiodiniaceae, *Ostreobium*, and other unclassified green algae (Chlorophyta), as the primary sugar exporters (**Supplementary data file 2**). All of these organisms perform the Calvin cycle, from which the intermediate glyceraldehyde-3-phosphate (G3P) serves as a precursor for

production of sugars including glucose, which is likely the main carbohydrate transported by SWEET proteins (*84*). Interestingly, we did not detect any bacterial transcripts expressing semiSWEET proteins—the bacterial equivalents of SWEET proteins—despite recent studies identifying sugar transporters in coral-associated bacteria (*26*). This suggests a potential specialisation within the coral holobiont, where eukaryotes may play a predominant role in carbohydrate-based energy exchange between symbionts.

We identified the expression of SWEET protein genes in *Ostreobium*, providing the first genomic and transcriptomic evidence of sugar export capabilities in skeleton-dwelling algae. This discovery suggests that *Ostreobium* can actively transport sugars outside its cells, making them potentially available for uptake by the coral host and other members of the coral microbiome. These results highlight a possible alternative energy source for corals, particularly under stress conditions when the loss of *Symbiodiniaceae* compromises the availability of photosynthates such as glucose.

We identified the expression of SWEET protein genes in *Ostreobium*, providing the first genomic and transcriptomic evidence of sugar export by skeleton-dwelling algae. This suggests that *Ostreobium* actively transports sugars into the coral skeleton, potentially making them available to the coral host and other members of the microbiome. These findings highlight a potential alternative carbon source for corals, particularly under stress conditions when *Symbiodiniaceae* are expelled, limiting the availability of photosynthates (*85*). Previous studies using 14C and 13C-labeled photoassimilates have demonstrated carbon translocation from endolithic algae into coral host lipids under both healthy and bleached conditions (*34*, *35*). Our findings provide molecular evidence that *Ostreobium* contributes to this process, underscoring its role in supporting coral energy demands during stress or bleaching events. This capacity for sugar export may represent a critical mechanism for maintaining coral resilience by mitigating energy starvation in the absence of *Symbiodiniaceae.*

Coral-associated fungi are increasingly recognized for their diverse roles, including influencing reef-scale biogeochemical cycles and shaping coral-associated and environmental microbiomes through chemical interactions (*86*). In this study, we identified expression of fungal transcripts encoding SWEET proteins, providing direct molecular evidence of fungi actively participating in sugar transport in the coral holobiont. This finding expands our understanding of their role in carbon cycling by demonstrating their contribution to fixed carbon transfer within the coral holobiont.

The identification of diverse eukaryotes capable of translocating sugars underscores their importance as a member of the coral probiotic. However, this capability may also pose risks, as sugar-exporting organisms could inadvertently support the proliferation of opportunistic or pathogenic bacteria, thereby potentially harming the coral. Therefore, further research is essential to elucidate the impacts of these sugar-translocating eukaryotes on coral health.

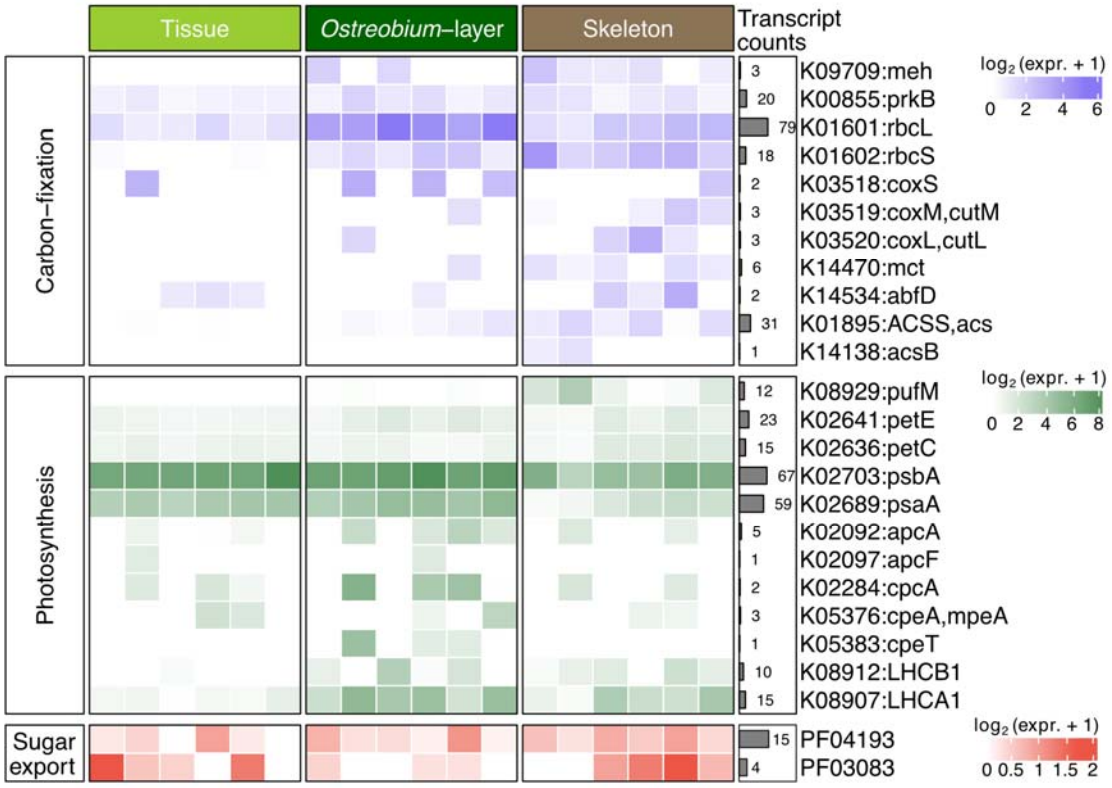

Fig. 2. Carbon Fixation, Photosynthesis and Sugar Export Across Coral Compartments. Heatmap displaying the expression of marker genes for carbon fixation photosynthesis, and SWEET protein-mediated sugar export across various coral compartments. Expression values for both were averaged for the count of transcripts and log2(expression + 1) transformed.

Skeleton dwelling microbes mediate nitrogen metabolism

Our analysis identified the expression of nitrogen-fixation marker genes (nifK, nifD, nifH) in both the Ostreobium layer and the deeper skeleton of *P. lutea*, indicating active nitrogen fixation in these anoxic environments (Fig. 3A). Nitrogen cycling within the coral holobiont is indispensable to reef ecology, enabling corals to thrive in nutrient-deficient waters through efficient nitrogen assimilation and recycling (*31*). While nitrogen-cycling microbes are consistently detected in coral-associated microbiota, (*31*, *87*) our study highlights

cyanobacteria as the primary nitrogen fixers in *P. lutea*. This finding contrasts earlier reports that heterotrophic bacteria dominate nitrogen fixation in corals (*88–90*) , aligning instead with recent genome-resolved metagenomic studies showing low diversity among nitrogen-fixing bacteria in this species (*24*, *25*). The predominance of cyanobacteria in nitrogen fixation emphasises their critical role in the nitrogen economy of the coral holobiont, particularly in supporting nitrogen-limited growth of Symbiodiniaceae (*9*, *91*). These results advance our understanding of the microbial interactions underpinning nutrient dynamics and overall reef health.

We also identified nitrification, the aerobic microbial process converting ammonia to hydroxylamine and subsequently to nitrite and nitrate, to be ubiquitous across all coral compartments (**Fig 3A**). This process is crucial for the coral holobiont as it reduces internal ammonium levels, thereby regulating the growth of Symbiodiniaceae and retaining dissolved inorganic nitrogen in an oxidised state, which is essential for preventing the accumulation of toxic ammonia and facilitating the availability of nitrogen for other metabolic processes (*31*). We identified active expression of the nitrification pathway by Thaumarchaeota, Gammaproteobacteria, and Chloroflexi (**Fig 3B and Fig 5**), particularly in the deeper skeleton, enhancing our understanding of the specific organisms involved and their roles in this process.

In contrast, denitrification was confined to the *Ostreobium* layer and deeper skeleton compartments, where we detected expression of the marker genes *napA, nirS, norB, and nosZ* (**Fig. 3A**). This localised expression indicates that denitrification, a key anaerobic process, primarily occurs in low-oxygen zones, particularly in deeper skeleton areas, where conditions are conducive to the reduction of nitrate to nitrogen gases. Nitrate reduction to nitrite via *NR* and *napA* was observed in both eukaryotic (Symbiodiniaceae and Ostreobium) and bacterial symbionts (including Rhodospirillales, Chlorobi, Proteobacteria, and Acidobacteria) (**Fig. 3A**; supplementary data file 3). The subsequent conversion of nitrite to nitric oxide, nitrous oxide, and nitrogen during denitrification was predominantly carried out by Thaumarchaeota, and Proteobacteria (α- and γ-), and Bacteroidota. Importantly, we identified fungal (Ascomycota) transcripts encoding the NIT-6 gene, responsible for reducing nitrite to ammonium via dissimilatory nitrate reduction to ammonium (DNRA), highlighting the role of fungi in nitrogen cycling in coral.

The interplay between nitrification and denitrification, particularly in the skeleton, is crucial for maintaining nitrogen homeostasis within the coral holobiont. The DNRA process, active under low-oxygen conditions (such as at night), converts nitrate back to ammonium, which

may be essential when nitrification, dependent on oxygen, is less active. This coupling between DNRA and nitrification ensures stable ammonium levels, which could promote coral growth. On the other hand, coupling denitrification and nitrification may help regulate nitrogen availability in nutrient-rich conditions, preventing excessive growth of Symbiodiniaceae and maintaining a balance within the coral ecosystem.

Our results significantly expand current understanding of nitrogen cycling within coral holobionts by identifying which microbial taxa are actively involved in these processes and how different compartments, including the coral skeleton, contribute to nitrogen dynamics. The skeleton, in particular, plays a critical role in both nitrification and denitrification, with different microbes in at different depths of the skeleton performing complementary functions that ensure nitrogen homeostasis within the coral host.

Sulphur metabolising microbes are widespread across coral compartments

Sulphur is readily available in seawater as inorganic sulphate and is assimilated by the coral host, Symbiodiniaceae, and diverse members of the coral microbiome into organic compounds, thus playing an essential role in the growth of coral holobionts (*92–94*). We identified expression of transcripts encoding for marker genes involved in sulphur cycling in all coral compartments (**Fig 3B**). The majority of sulphur cycling marker genes exhibited higher expression levels in the skeleton, suggesting a predominant role of the endolithic microbiome. Anaerobic dissimilatory sulphate reduction marker genes (*apr*A and *apr*B) were exclusively expressed in the coral skeleton, with taxonomic analysis identifying them as known sulphate-reducing bacteria (SRB), specifically Deltaproteobacteria and anoxygenic phototrophic Chlorobi (**Fig 3B**). It is important to note that while Chlorobi possess *apr*A and *apr*B genes, they are primarily involved in sulphur oxidation rather than sulphate reduction (*95*, *96*). Metabarcoding studies have predicted abundant sulphate reducers in the coral skeleton, and recent metagenomic studies have assembled genomes of SRBs from the skeleton of *P. lutea* and *I. palifera (24)*. The coral skeleton not only harbours SRBs but also anoxygenic phototrophs such as Chlorobi, supporting the presence of anoxic microniches in the coral skeleton (*14*, *24*, *27*, *76*). SRB and Chlorobi have also been shown to form a syntrophic relationship, where sulphide produced by SRB is used as an electron donor for photosynthetic Chlorobi in microbial mats, deeper lake waters and coral skeleton (*27*, *97–99*). Our identification of transcripts from SRBs and Chlorobi within the same compartment of the holobiont provides molecular evidence for such a syntrophic relationship in the coral skeleton.

We observed a complex expression profile of assimilatory sulphate reduction marker genes

(*cys*C, *cys*I, *cys*J, and SUOX), which were expressed in all three compartments; with the exception of *cys*D, which was exclusively expressed in the deep skeleton layer (**Fig 3B**). Additionally, the gene encoding the enzyme *cys*K, crucial for linking assimilatory sulphate reduction with the biosynthesis of the essential amino acid L-Cysteine, was consistently expressed across all compartments (**Fig 3B**). Taxonomic analysis of transcripts encoding these ASR marker genes identified a diverse range of symbiont members, including microeukaryotes such as Symbiodiniaceae, *Ostreobium* and other Chlorophyta, as well as prokaryotic groups Cyanobacteria, Thaumarchaeota, and Proteobacteria (**Fig 3B**). The identification of transcripts encoding ASR exclusively annotated to algae and bacteria aligns with the understanding that this pathway is restricted to plants, algae and microbes(*100*).

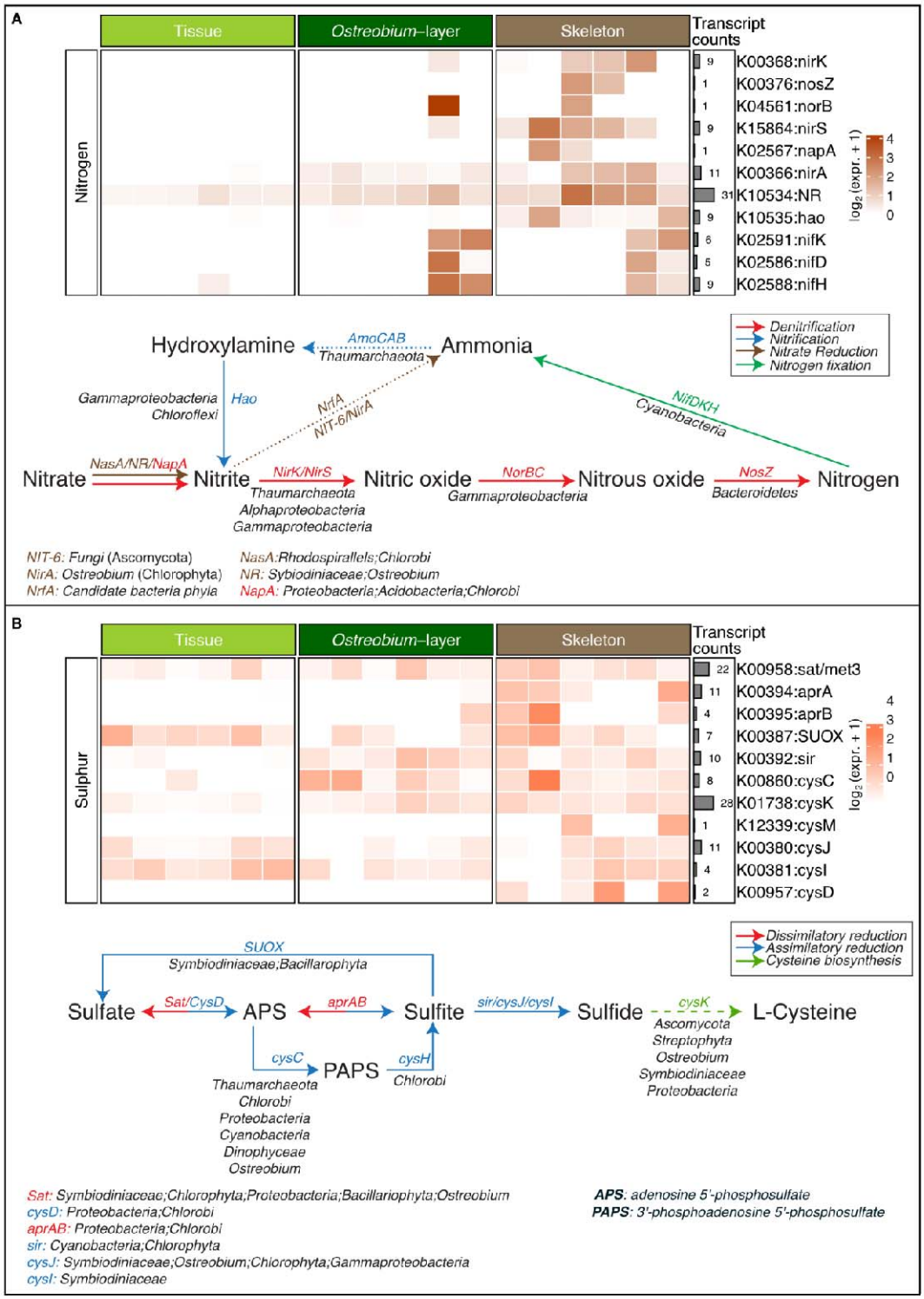

Fig 3. Transcriptome- based expression profiles of Nitrogen and Sulfur metabolism across coral compartments. A) Heatmap showing the expression profiles of marker genes involved in nitrogen metabolism across coral compartments, along with a bar plot indicating the count of transcripts. The nitrogen cycle is illustrated, highlighting subpathways and contributing community members. B) Heatmap showing the expression profiles of marker genes involved in sulphur metabolism across coral compartments, along with a bar plot

indicating the count of transcripts. The sulphur metabolism pathway is illustrated, highlighting subpathways and contributing community members. Expression values for both were averaged and log2(expression + 1) transformed.

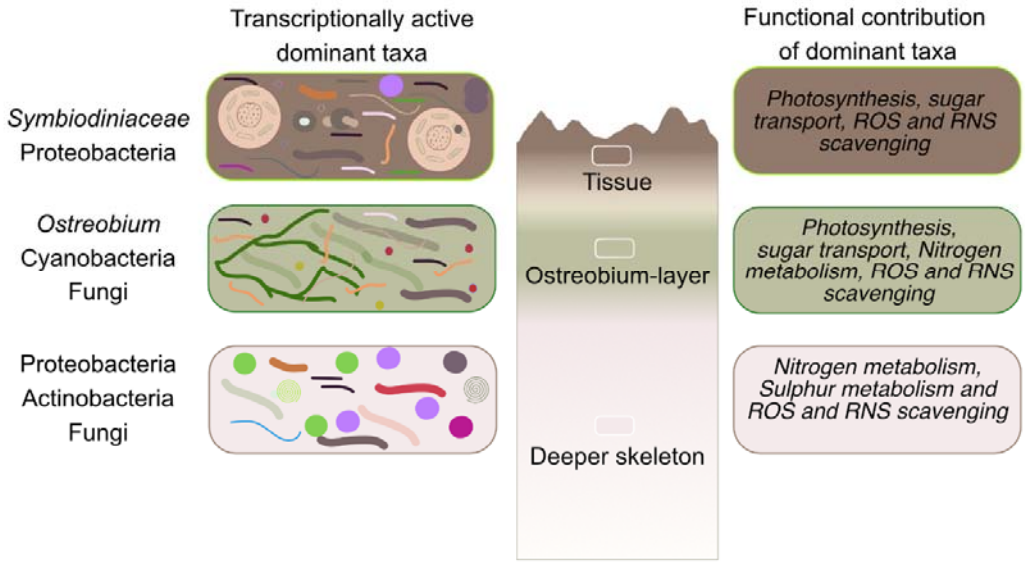

Figure 5. Overview of the transcriptionally dominant taxa and their functional contribution in different compartments of the coral identified in this study.

Microeukaryotes are the major scavengers of ROS and RNS in *P. lutea*

Coral reefs are facing unprecedented threats due to rising sea surface temperatures, resulting in more frequent and prolonged coral bleaching events (*37*). Most theories on the onset of coral bleaching suggest that the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a key trigger, leading to cellular damage and stress under adverse environmental conditions (*101*). Rapid scavenging of ROS and RNS is thus crucial in maintaining homeostasis and protecting the coral holobiont in stressed and healthy corals. We observed widespread expression of seven protein families with functions related to ROS or RNS scavenging across the tissue, *Ostreobium* layer, and deeper skeleton compartments. Specifically, transcripts encoding for genes involved in RNS scavenging, including nitric oxide reductases (PF00175: *hmp*; PF00115 and PF00034: *nor*BC) and peroxynitrite to nitrate reduction (PF10417: *ahp*C) were expressed in all compartments (**Fig 4**). A single transcript encoding nitrous oxide reductase (PF18764: *nos*Z) was identified, and expressed exclusively in deeper skeleton samples. This enzyme catalyses the last step of the dissimilatory metabolic pathway of denitrifying bacteria, which were abundant in the deeper skeleton. Similar observations were made for ROS scavenging expression profiles.

We identified 48, 43, and 41 transcripts encoding the general ROS scavenger Glutaredoxin (PF00462), as well as hydrogen peroxide scavengers Peroxiredoxins (PF00578) and peroxidases (PF00141). These transcripts were expressed in all coral compartments along with transcripts encoding superoxide dismutases (PF00081 and PF02777: SOD Mn and PF00080:SOD cu-zn) (**Fig 4**). The majority of ROS and RNS scavenging transcripts were of micro-eukaryotic origin (76%)**,** including Symbiodiniaceae, *Ostreobium*, Chlorophyta, and Fungi, while others were from archaeal and bacterial sources (24%), encompassing Thaumarchaeota, Proteobacteria (Rhodospirillales, Xanthomonadales), Cyanobacteria, Chlorobi, Chloroflexi and Bacteroidetes (*Candidatus* Amoebophilus) (**Supplementary data file 4**).

Scavenging reactive oxygen (ROS) and nitrogen species (RNS) is a key function of bacterial candidates for coral probiotics, as emphasised in recent studies identifying potential probiotic bacteria for corals (*2*, *26*, *102–104*). However, the identification of the majority of ROS and RNS scavenging transcripts in micro-eukaryotic inhabitants of the coral holobiont rather than in bacteria, raises the question of which organisms are the main players in scavenging ROS and RNS under thermal stress. This finding suggests that current strategies for designing coral probiotics, which primarily focus on bacterial functions, should be refined through additional molecular and experimental validation to provide functional insights. These insights could guide the assembly of a more comprehensive consortium of beneficial symbionts for corals.

Fig. 4. Transcriptome-based expression profiles of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) marker genes across coral compartments. Heatmap displaying the expression of different protein families, count of transcripts for each family involved in ROS and RNS scavenging across coral compartments. Expression values for both were averaged and log2(expression + 1) transformed.

Conclusion

This study provides novel insights into the transcriptionally active communities within the coral holobiont, with a focus on their functional roles across distinct ecological compartments: coral tissue, the endolithic *Ostreobium* layer, and the deeper skeleton layer. Our analysis revealed a nuanced stratification of microbial activity, with microeukaryotes dominating key metabolic processes in the coral tissue and *Ostreobium* layer, while prokaryotes emerge as the primary active symbionts in the deeper skeleton (**Fig 5**). These findings underscore the profound influence of physicochemical gradients—including light penetration, nutrient availability, and carbon distribution—in sculpting both community composition and functional specialization within the holobiont. Functional analysis highlighted the critical contributions of *Symbiodiniaceae, Ostreobium*, and Cyanobacteria to photosynthate production, with *Symbiodiniaceae* predominantly occupying the coral tissue, while *Ostreobium* and Cyanobacteria play pivotal roles in the endolithic *Ostreobium*-layer (**Fig 5**). These symbionts, evolutionarily adapted to varying light conditions and utilizing distinct photosynthetic pigments, demonstrated unique nutrient cycling capabilities. Notably, in addition to *Symbiodiniaceae*, we identified *Ostreobium* and fungi as the only taxa capable of exporting sugars via SWEET proteins, providing novel insights into nutrient exchange mechanisms within the holobiont. Analysis of nitrogen metabolic pathways revealed Cyanobacteria as the exclusive nitrogen fixers, with other nitrogen and sulphur metabolic

processes likely distributed across holobiont members, suggesting a robust functional redundancy that potentially enhances ecosystem resilience (**Fig 5**). Our analysis also identified microeukaryotes as the primary scavengers of ROS and RNS across all compartments, playing a crucial role in maintaining holobiont health, particularly under thermal stress conditions (**Fig 5**). These findings offer a comprehensive view of the metabolic activities within the coral microbiome, elucidating the intricate functional roles of its diverse members. While this research significantly advances our understanding of coral holobiont functions, it simultaneously highlights the complexity of these systems and the necessity for more targeted research focussed on the microenvironmental niches of the coral holobiont. Future investigations should employ advanced sampling techniques and high-resolution analytical methods to more precisely map the functional dynamics between coral hosts, their symbionts, and the surrounding microenvironment (*11*, *105*, *106*). Ultimately, the insights gained from this study are invaluable for advancing coral reef conservation efforts. By identifying functionally relevant symbiotic members, we can better inform the design of coral probiotics and other strategies aimed at enhancing coral resilience in the face of climate change and other environmental stressors.

Data and code availability

Raw reads generated in this study have been submitted to NCBI SRA under the Bioproject PRJNA1207708. All codes and files required to regenerate the figures have been made available via github.

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References

1. C. Jaspers, S. Fraune, A. E. Arnold, D. J. Miller, T. C. G. Bosch, C. R. Voolstra, Consortium of Australian Academy of Science Boden Research Conference Participants, Resolving structure and function of metaorganisms through a holistic framework combining reductionist and integrative approaches. *Zoology* **133**, 81–87 (2019).

2. R. S. Peixoto, M. Sweet, H. D. M. Villela, P. Cardoso, T. Thomas, C. R. Voolstra, L. Høj, D. G. Bourne, Coral Probiotics: Premise, Promise, Prospects. *Annu Rev Anim Biosci* **9**, 265–288 (2021).

3. M. J. H. van Oppen, L. L. Blackall, Coral microbiome dynamics, functions and design in a changing world. *Nat. Rev. Microbiol.* **17**, 557–567 (2019).

4. E. Rosenberg, O. Koren, L. Reshef, R. Efrony, I. Zilber-Rosenberg, The role of microorganisms in coral health, disease and evolution. *Nat. Rev. Microbiol.* **5**, 355–362 (2007).

5. F. Rohwer, V. Seguritan, F. Azam, N. Knowlton, Diversity and distribution of coral-associated bacteria. *Mar. Ecol. Prog. Ser.* **243**, 1–10 (2002).

6. R. V. Thurber, J. P. Payet, A. R. Thurber, A. M. S. Correa, Virus-host interactions and their roles in coral reef health and disease. *Nat. Rev. Microbiol.* **15**, 205–216 (2017).

7. T. C. LaJeunesse, J. E. Parkinson, P. W. Gabrielson, H. J. Jeong, J. D. Reimer, C. R. Voolstra, S. R. Santos, Systematic Revision of Symbiodiniaceae Highlights the Antiquity and Diversity of Coral Endosymbionts. *Curr. Biol.* **28**, 2570–2580.e6 (2018).

8. L. Muscatine, The role of symbiotic algae in carbon and energy flux in reef corals. *Ecosystems of the world* **25**, 75–87 (1990).

9. L. A. Morris, C. R. Voolstra, K. M. Quigley, D. G. Bourne, L. K. Bay, Nutrient Availability and Metabolism Affect the Stability of Coral-Symbiodiniaceae Symbioses. *Trends Microbiol.* **27**, 678–689 (2019).

10. M. J. Huggett, A. Apprill, Coral microbiome database: Integration of sequences reveals high diversity and relatedness of coral-associated microbes. *Environ. Microbiol. Rep.* **11**, 372–385 (2019).

11. E. Bollati, D. J. Hughes, D. J. Suggett, J.-B. Raina, M. Kühl, Microscale sampling of the coral gastrovascular cavity reveals a gut-like microbial community. *anim microbiome* **6** (2024).

12. M. J. Sweet, A. Croquer, J. C. Bythell, Bacterial assemblages differ between compartments within the coral holobiont. *Coral Reefs* **30**, 39–52 (2011).

13. V. R. Marcelino, M. J. van Oppen, H. Verbruggen, Highly structured prokaryote communities exist within the skeleton of coral colonies. *ISME J.* **12**, 300–303 (2018).

14. F. Ricci, K. Tandon, M. Moßhammer, E. H.-J. Cho, L. L. Blackall, M. Kühl, H. Verbruggen, Fine-scale mapping of physicochemical and microbial landscapes of the coral skeleton. *Environ. Microbiol.* **25**, 1505–1521 (2023).

15. K. Tandon, M. M. Pasella, C. Iha, F. Ricci, J. Hu, C. J. O'Kelly, M. Medina, M. Kühl, H. Verbruggen, Every refuge has its price: Ostreobium as a model for understanding how algae can live in rock and stay in business. *Semin. Cell Dev. Biol.* **134**, 27–36 (2023).

16. A. M. Bonacolta, B. A. Weiler, T. Porta-Fitó, M. Sweet, P. Keeling, J. del Campo,

Beyond the Symbiodiniaceae: diversity and role of microeukaryotic coral symbionts. *Coral Reefs* **42**, 567–577 (2023).

17. M. McCauley, T. L. Goulet, C. R. Jackson, S. Loesgen, Systematic review of cnidarian microbiomes reveals insights into the structure, specificity, and fidelity of marine associations. *Nat. Commun.* **14**, 4899 (2023).

18. R. Rowan, D. A. Powers, Ribosomal RNA sequences and the diversity of symbiotic dinoflagellates (zooxanthellae). *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3639–3643 (1992).

19. F. J. Pollock, R. McMinds, S. Smith, D. G. Bourne, B. L. Willis, M. Medina, R. V. Thurber, J. R. Zaneveld, Coral-associated bacteria demonstrate phylosymbiosis and cophylogeny. *Nat. Commun.* **9**, 4921 (2018).

20. L. Fujise, D. J. Suggett, M. Stat, T. Kahlke, M. Bunce, S. G. Gardner, S. Goyen, S. Woodcock, P. J. Ralph, J. R. Seymour, N. Siboni, M. R. Nitschke, Unlocking the phylogenetic diversity, primary habitats, and abundances of free-living Symbiodiniaceae on a coral reef. *Mol. Ecol.* **30**, 343–360 (2021).

21. L. K. Lee, C. P. Leaw, L. C. Lee, Z. F. Lim, K. S. Hii, A. A. Chan, H. Gu, P. T. Lim, Molecular diversity and assemblages of coral symbionts (Symbiodiniaceae) in diverse scleractinian coral species. *Mar. Environ. Res.* **179**, 105706 (2022).

22. V. R. Marcelino, K. M. Morrow, M. J. H. van Oppen, D. G. Bourne, H. Verbruggen, Diversity and stability of coral endolithic microbial communities at a naturally high pCO2 reef. *Mol. Ecol.* **26**, 5344–5357 (2017).

23. F. Ricci, A. Fordyce, W. Leggat, L. L. Blackall, T. Ainsworth, H. Verbruggen, Multiple techniques point to oxygenic phototrophs dominating the Isopora palifera skeletal microbiome. *Coral Reefs* **40**, 275–282 (2021).

24. K. Tandon, F. Ricci, J. Costa, M. Medina, M. Kühl, L. L. Blackall, H. Verbruggen, Genomic view of the diversity and functional role of archaea and bacteria in the skeleton of the reef-building corals Porites lutea and Isopora palifera. *Gigascience* **12** (2022).

25. S. J. Robbins, C. M. Singleton, C. X. Chan, L. F. Messer, A. U. Geers, H. Ying, A. Baker, S. C. Bell, K. M. Morrow, M. A. Ragan, D. J. Miller, S. Forêt, ReFuGe2020 Consortium, C. R. Voolstra, G. W. Tyson, D. G. Bourne, A genomic view of the reef-building coral Porites lutea and its microbial symbionts. *Nat Microbiol* **4**, 2090–2100 (2019).

26. T. Doering, K. Tandon, S. H. Topa, S. J. Pidot, L. L. Blackall, M. J. H. van Oppen, Genomic exploration of coral-associated bacteria: identifying probiotic candidates to increase coral bleaching resilience in Galaxea fascicularis. *Microbiome* **11**, 185 (2023).

27. S.-H. Yang, K. Tandon, C.-Y. Lu, N. Wada, C.-J. Shih, S. S.-Y. Hsiao, W.-N. Jane, T.-C. Lee, C.-M. Yang, C.-T. Liu, V. Denis, Y.-T. Wu, L.-T. Wang, L. Huang, D.-C. Lee, Y.-W. Wu, H. Yamashiro, S.-L. Tang, Metagenomic, phylogenetic, and functional characterization of predominant endolithic green sulfur bacteria in the coral Isopora palifera. *Microbiome* **7**, 3 (2019).

28. M. Sweet, H. Villela, T. Keller-Costa, R. Costa, S. Romano, D. G. Bourne, A. Cárdenas, M. J. Huggett, A. H. Kerwin, F. Kuek, M. Medina, J. L. Meyer, M. Müller, F. J. Pollock, M. S. Rappé, M. Sere, K. H. Sharp, C. R. Voolstra, N. Zaccardi, M. Ziegler, R. Peixoto, Insights into the Cultured Bacterial Fraction of Corals. *mSystems* **6**, e0124920 (2021).

29. P. G. Falkowski, Z. Dubinsky, L. Muscatine, J. W. Porter, Light and the Bioenergetics of a Symbiotic Coral. *Bioscience* **34**, 705–709 (1984).

30. K. Tandon, C.-Y. Lu, P.-W. Chiang, N. Wada, S.-H. Yang, Y.-F. Chan, P.-Y. Chen, H.-Y. Chang, Y.-J. Chiou, M.-S. Chou, W.-M. Chen, S.-L. Tang, Comparative genomics: Dominant coral-bacterium Endozoicomonas acroporae metabolizes dimethylsulfoniopropionate (DMSP). *ISME J.* **14**, 1290–1303 (2020).

31. N. Rädecker, C. Pogoreutz, C. R. Voolstra, J. Wiedenmann, C. Wild, Nitrogen cycling in corals: the key to understanding holobiont functioning? *Trends Microbiol.* **23**, 490–497 (2015).

32. M. J. Neave, C. T. Michell, A. Apprill, C. R. Voolstra, Endozoicomonas genomes reveal functional adaptation and plasticity in bacterial strains symbiotically associated with diverse marine hosts. *Sci. Rep.* **7**, 40579 (2017).

33. D. Schlichter, H. Kampmann, S. Conrady, Trophic potential and photoecology of endolithic algae living within coral skeletons. *Mar. Ecol.* **18**, 299–317 (1997).

34. M. Fine, Y. Loya, Endolithic algae: an alternative source of photoassimilates during coral bleaching. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **269**, 1205–1210 (2002).

35. L. Sangsawang, B. E. Casareto, H. Ohba, H. M. Vu, A. Meekaew, T. Suzuki, T. Yeemin, Y. Suzuki, 13C and 15N assimilation and organic matter translocation by the endolithic community in the massive coral Porites lutea. *R Soc Open Sci* **4**, 171201 (2017).

36. E. A. Ottesen, C. R. Young, S. M. Gifford, J. M. Eppley, R. Marin 3rd, S. C. Schuster, C. A. Scholin, E. F. DeLong, Ocean microbes. Multispecies diel transcriptional oscillations in open ocean heterotrophic bacterial assemblages. *Science* **345**, 207–212 (2014).

37. T. P. Hughes, J. T. Kerry, S. R. Connolly, J. G. Álvarez-Romero, C. M. Eakin, S. F. Heron, M. A. Gonzalez, J. Moneghetti, Emergent properties in the responses of tropical corals to recurrent climate extremes. *Curr. Biol.* **31**, 5393–5399.e3 (2021).

38. M. J. H. van Oppen, J. K. Oliver, H. M. Putnam, R. D. Gates, Building coral reef resilience through assisted evolution. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 2307–2313 (2015).

39. A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).

40. E. Kopylova, L. Noé, H. Touzet, SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* **28**, 3211–3217 (2012).

41. P. Yilmaz, L. W. Parfrey, P. Yarza, J. Gerken, E. Pruesse, C. Quast, T. Schweer, J. Peplies, W. Ludwig, F. O. Glöckner, The SILVA and “All-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Res.* **42**, D643–D648 (2013).

42. H. R. Gruber-Vodicka, B. K. B. Seah, E. Pruesse, phyloFlash: Rapid Small-Subunit rRNA Profiling and Targeted Assembly from Metagenomes. *mSystems* **5** (2020).

43. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).

44. H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, 1000 Genome Project Data Processing Subgroup, The Sequence

Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).

45. M. G. Grabherr, B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644–652 (2011).

46. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).

47. B. Buchfink, K. Reuter, H.-G. Drost, Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat. Methods* **18**, 366–368 (2021).

48. D. H. Huson, S. Beier, I. Flade, A. Górska, M. El-Hadidi, S. Mitra, H.-J. Ruscheweyh, R. Tappu, MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLoS Comput. Biol.* **12**, e1004957 (2016).

49. B. Li, C. N. Dewey, RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).

50. P. J. McMurdie, S. Holmes, phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* **8**, e61217 (2013).

51. H. Wickham, ggplot2. *Wiley Interdiscip. Rev. Comput. Stat.* **3**, 180–185 (2011).

52. D. M. Bryant, K. Johnson, T. DiTommaso, T. Tickle, M. B. Couger, D. Payzin-Dogru, T. J. Lee, N. D. Leigh, T.-H. Kuo, F. G. Davis, J. Bateman, S. Bryant, A. R. Guzikowski, S. L. Tsai, S. Coyne, W. W. Ye, R. M. Freeman Jr, L. Peshkin, C. J. Tabin, A. Regev, B. J. Haas, J. L. Whited, A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. *Cell Rep.* **18**, 762–776 (2017).

53. C. P. Cantalapiedra, A. Hernández-Plaza, I. Letunic, P. Bork, J. Huerta-Cepas, eggNOG-mapper v2: Functional Annotation, Orthology Assignments, and Domain Prediction at the Metagenomic Scale. *Mol. Biol. Evol.* **38**, 5825–5829 (2021).

54. J. Huerta-Cepas, D. Szklarczyk, D. Heller, A. Hernández-Plaza, S. K. Forslund, H. Cook, D. R. Mende, I. Letunic, T. Rattei, L. J. Jensen, C. von Mering, P. Bork, eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.* **47**, D309–D314 (2019).

55. M. Kanehisa, Y. Sato, K. Morishima, BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J. Mol. Biol.* **428**, 726–731 (2016).

56. P. Jones, D. Binns, H.-Y. Chang, M. Fraser, W. Li, C. McAnulla, H. McWilliam, J. Maslen, A. Mitchell, G. Nuka, S. Pesseat, A. F. Quinn, A. Sangrador-Vegas, M. Scheremetjew, S.-Y. Yong, R. Lopez, S. Hunter, InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30**, 1236–1240 (2014).

57. G. Salazar, L. Paoli, A. Alberti, J. Huerta-Cepas, H.-J. Ruscheweyh, M. Cuenca, C. M. Field, L. P. Coelho, C. Cruaud, S. Engelen, A. C. Gregory, K. Labadie, C. Marec, E. Pelletier, M. Royo-Llonch, S. Roux, P. Sánchez, H. Uehara, A. A. Zayed, G. Zeller, M. Carmichael, C. Dimier, J. Ferland, S. Kandels, M. Picheral, S. Pisarev, J. Poulain, Tara Oceans Coordinators, S. G. Acinas, M. Babin, P. Bork, C. Bowler, C. de Vargas, L. Guidi, P. Hingamp, D. Iudicone, L. Karp-Boss, E. Karsenti, H. Ogata, S. Pesant, S.

Speich, M. B. Sullivan, P. Wincker, S. Sunagawa, Gene Expression Changes and Community Turnover Differentially Shape the Global Ocean Metatranscriptome. *Cell* **179**, 1068–1083.e21 (2019).

58. Z. Gu, R. Eils, M. Schlesner, Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**, 2847–2849 (2016).

59. L. M. van der Loos, R. Nijland, Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. *Mol. Ecol.* **30**, 3270–3288 (2021).

60. S. K. Davy, D. Allemand, V. M. Weis, Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol. Mol. Biol. Rev.* **76**, 229–261 (2012).

61. W. K. Kwong, J. Del Campo, V. Mathur, M. J. A. Vermeij, P. J. Keeling, A widespread coral-infecting apicomplexan with chlorophyll biosynthesis genes. *Nature* **568**, 103–107 (2019).

62. A. Oren, G. M. Garrity, Valid publication of the names of forty-two phyla of prokaryotes. *Int. J. Syst. Evol. Microbiol.* **71** (2021).

63. A. Cárdenas, J.-B. Raina, C. Pogoreutz, N. Rädecker, J. Bougoure, P. Guagliardo, M. Pernice, C. R. Voolstra, Greater functional diversity and redundancy of coral endolithic microbiomes align with lower coral bleaching susceptibility. *ISME J.* **16**, 2406–2420 (2022).

64. J. R. Guest, J. Low, K. Tun, B. Wilson, C. Ng, D. Raingeard, K. E. Ulstrup, J. T. I. Tanzil, P. A. Todd, T. C. Toh, D. McDougald, L. M. Chou, P. D. Steinberg, Coral community response to bleaching on a highly disturbed reef. *Sci. Rep.* **6**, 20717 (2016).

65. P. Buerger, G. M. Schmidt, M. Wall, C. Held, C. Richter, Temperature tolerance of the coral Porites lutea exposed to simulated large amplitude internal waves (LAIW). *J. Exp. Mar. Bio. Ecol.* **471**, 232–239 (2015).

66. J. Peng, C.-E. Wegner, Q. Bei, P. Liu, W. Liesack, Metatranscriptomics reveals a differential temperature effect on the structural and functional organization of the anaerobic food web in rice field soil. *Microbiome* **6**, 169 (2018).

67. N. Vannier, F. Mesny, F. Getzke, G. Chesneau, L. Dethier, J. Ordon, T. Thiergart, S. Hacquard, Genome-resolved metatranscriptomics reveals conserved root colonization determinants in a synthetic microbiota. *Nat. Commun.* **14**, 8274 (2023).

68. S. R. Law, A. R. Serrano, Y. Daguerre, J. Sundh, A. N. Schneider, Z. R. Stangl, D. Castro, M. Grabherr, T. Näsholm, N. R. Street, V. Hurry, Metatranscriptomics captures dynamic shifts in mycorrhizal coordination in boreal forests. *Proc. Natl. Acad. Sci. U. S. A.* **119**, e2118852119 (2022).

69. S. Campana, A. Riesgo, E. Jongepier, J. Fuss, G. Muyzer, J. M. de Goeij, Meta-transcriptomic comparison of two sponge holobionts feeding on coral- and macroalgal-dissolved organic matter. *BMC Genomics* **23**, 674 (2022).

70. A. Schoenle, M. Hohlfeld, K. Hermanns, F. Mahé, C. de Vargas, F. Nitsche, H. Arndt, High and specific diversity of protists in the deep-sea basins dominated by diplonemids, kinetoplastids, ciliates and foraminiferans. *Commun Biol* **4**, 501 (2021).

71. S. L. Pichard, L. Campbell, J. H. Paul, Diversity of the ribulose bisphosphate carboxylase/oxygenase form I gene (rbcL) in natural phytoplankton communities. *Appl.*

Environ. Microbiol. **63**, 3600–3606 (1997).

72. B. K. Swan, M. Martinez-Garcia, C. M. Preston, A. Sczyrba, T. Woyke, D. Lamy, T. Reinthaler, N. J. Poulton, E. D. P. Masland, M. L. Gomez, M. E. Sieracki, E. F. DeLong, G. J. Herndl, R. Stepanauskas, Potential for chemolithoautotrophy among ubiquitous bacteria lineages in the dark ocean. *Science* **333**, 1296–1300 (2011).

73. J. A. Raven, Contributions of anoxygenic and oxygenic phototrophy and chemolithotrophy to carbon and oxygen fluxes in aquatic environments. *Aquat. Microb. Ecol.* **56**, 177–192 (2009).

74. F. Ricci, C. Greening, Chemosynthesis: a neglected foundation of marine ecology and biogeochemistry: (Trends in Microbiology, Published online January 30, 2024). *Trends Microbiol.* **32**, 723 (2024).

75. M. P. Lesser, M. Sabrina Pankey, M. Slattery, K. J. Macartney, D. J. Gochfeld, Microbiome diversity and metabolic capacity determines the trophic ecology of the holobiont in Caribbean sponges. *ISME Commun.* **2**, 112 (2022).

76. M. Kühl, G. Holst, A. W. D. Larkum, P. J. Ralph, IMAGING OF OXYGEN DYNAMICS WITHIN THE ENDOLITHIC ALGAL COMMUNITY OF THE MASSIVE CORAL PORITES LOBATA(1). *J. Phycol.* **44**, 541–550 (2008).

77. R. Samsonoff Wa Maccoll, Biliproteins and phycobilisomes from cyanobacteria and red algae at the extreme habitats. *Arch* **176**, 400–405 (2001).

78. A. de los Ríos, M. Grube, L. G. Sancho, C. Ascaso, Ultrastructural and genetic characteristics of endolithic cyanobacterial biofilms colonizing Antarctic granite rocks: Antarctic endolithic cyanobacterial biofilms. *FEMS Microbiol. Ecol.* **59**, 386–395 (2007).

79. S. H. Magnusson, M. Fine, M. Kühl, Light microclimate of endolithic phototrophs in the scleractinian corals Montipora monasteriata and Porites cylindrica. *Mar. Ecol. Prog. Ser.* **332**, 119–128 (2007).

80. C. Iha, K. E. Dougan, J. A. Varela, V. Avila, C. J. Jackson, K. A. Bogaert, Y. Chen, L. M. Judd, R. Wick, K. E. Holt, M. M. Pasella, F. Ricci, S. I. Repetti, M. Medina, V. R. Marcelino, C. X. Chan, H. Verbruggen, Genomic adaptations to an endolithic lifestyle in the coral-associated alga Ostreobium. *Curr. Biol.* **31**, 1393–1402.e5 (2021).

81. M. Lichtenberg, A. W. D. Larkum, M. Kühl, Photosynthetic Acclimation of Symbiodinium in hospite Depends on Vertical Position in the Tissue of the Scleractinian Coral Montastrea curta. *Front. Microbiol.* **7**, 230 (2016).

82. O. Hoegh-Guldberg, P. J. Mumby, A. J. Hooten, R. S. Steneck, P. Greenfield, E. Gomez, C. D. Harvell, P. F. Sale, A. J. Edwards, K. Caldeira, N. Knowlton, C. M. Eakin, R. Iglesias-Prieto, N. Muthiga, R. H. Bradbury, A. Dubi, M. E. Hatziolos, Coral reefs under rapid climate change and ocean acidification. *Science* **318**, 1737–1742 (2007).

83. N. Rädecker, C. Pogoreutz, H. M. Gegner, A. Cárdenas, F. Roth, J. Bougoure, P. Guagliardo, C. Wild, M. Pernice, J.-B. Raina, A. Meibom, C. R. Voolstra, Heat stress destabilizes symbiotic nutrient cycling in corals. *Proc. Natl. Acad. Sci. U. S. A.* **118** (2021).

84. J. Ji, L. Yang, Z. Fang, Y. Zhang, M. Zhuang, H. Lv, Y. Wang, Plant SWEET Family of Sugar Transporters: Structure, Evolution and Biological Functions. *Biomolecules* **12** (2022).

85. O. Hoegh-Guldberg, Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshwater Res.* **50**, 839–866 (1999).

86. A. Roik, M. Reverter, C. Pogoreutz, A roadmap to understanding diversity and function of coral reef-associated fungi. *FEMS Microbiol. Rev.* **46** (2022).

87. A. R. Mohamed, M. A. Ochsenkühn, A. M. Kazlak, A. Moustafa, S. A. Amin, The coral microbiome: towards an understanding of the molecular mechanisms of coral-microbiota interactions. *FEMS Microbiol. Rev.* **47** (2023).

88. N. D. Olson, T. D. Ainsworth, R. D. Gates, M. Takabayashi, Diazotrophic bacteria associated with Hawaiian Montipora corals: Diversity and abundance in correlation with symbiotic dinoflagellates. *J. Exp. Mar. Bio. Ecol.* **371**, 140–146 (2009).

89. K. A. Lema, B. L. Willis, D. G. Bourne, Amplicon pyrosequencing reveals spatial and temporal consistency in diazotroph assemblages of the Acropora millepora microbiome. *Environ. Microbiol.* **16**, 3345–3359 (2014).

90. K. A. Lema, D. G. Bourne, B. L. Willis, Onset and establishment of diazotrophs and other bacterial associates in the early life history stages of the coral Acropora millepora. *Mol. Ecol.* **23**, 4682–4695 (2014).

91. C. Cook, C. D'Elia, Are natural populations of zooxanthellae ever nutrient-limited? *Symbiosis* **4**, 199–211 (1987).

92. J. Li, Q. Yang, J. Dong, M. Sweet, Y. Zhang, C. Liu, Y. Zhang, X. Tang, W. Zhang, S. Zhang, Microbiome Engineering: A Promising Approach to Improve Coral Health. *Proc. Est. Acad. Sci. Eng.* **28**, 105–116 (2023).

93. M. A. Moran, B. P. Durham, Sulfur metabolites in the pelagic ocean. *Nat. Rev. Microbiol.* **17**, 665–678 (2019).

94. T. Higuchi, K. Tanaka, K. Shirai, I. Yuyama, T. Mezaki, N. Takahata, Y. Sano, Sulfur assimilation in corals with aposymbiotic and symbiotic zooxanthellae. *Environ. Microbiol. Rep.* **13**, 98–103 (2021).

95. B. Meyer, J. Kuever, Homology modeling of dissimilatory APS reductases (AprBA) of sulfur-oxidizing and sulfate-reducing prokaryotes. *PLoS One* **3**, e1514 (2008).

96. T. Watanabe, H. Kojima, M. Fukui, Identity of major sulfur-cycle prokaryotes in freshwater lake ecosystems revealed by a comprehensive phylogenetic study of the dissimilatory adenylylsulfate reductase. *Sci. Rep.* **6**, 36262 (2016).

97. J. F. Imhoff, Biology of green sulfur bacteria, *eLS* (2014). https://doi.org/10.1002/9780470015902.a0000458.pub2.

98. C. Ng, M. Z. DeMaere, T. J. Williams, F. M. Lauro, M. Raftery, J. A. E. Gibson, C. Andrews-Pfannkoch, M. Lewis, J. M. Hoffman, T. Thomas, R. Cavicchioli, Metaproteogenomic analysis of a dominant green sulfur bacterium from Ace Lake, Antarctica. *ISME J.* **4**, 1002–1019 (2010).

99. Y.-H. Chen, S.-H. Yang, K. Tandon, C.-Y. Lu, H.-J. Chen, C.-J. Shih, S.-L. Tang, Potential syntrophic relationship between coral-associated Prosthecochloris and its companion sulfate-reducing bacterium unveiled by genomic analysis. *Microb Genom* **7** (2021).

100. I. Kushkevych, J. Cejnar, J. Treml, D. Dordević, P. Kollar, M. Vítězová, Recent

Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria. *Cells* **9** (2020).

101. J. Helgoe, S. K. Davy, V. M. Weis, M. Rodriguez-Lanetty, Triggers, cascades, and endpoints: connecting the dots of coral bleaching mechanisms. *Biol. Rev. Camb. Philos. Soc.* **99**, 715–752 (2024).

102. A. M. Dungan, D. Bulach, H. Lin, M. J. H. van Oppen, L. L. Blackall, Development of a free radical scavenging bacterial consortium to mitigate oxidative stress in cnidarians. *Microb. Biotechnol.* **14**, 2025–2040 (2021).

103. N. Delgadillo-Ordoñez, N. Garcias-Bonet, I. Raimundo, F. C. García, H. Villela, E. O. Osman, E. P. Santoro, J. Curdia, J. G. D. Rosado, P. Cardoso, A. Alsaggaf, A. Barno, C. P. Antony, C. Bocanegra, M. L. Berumen, C. R. Voolstra, F. Benzoni, S. Carvalho, R. S. Peixoto, Probiotics reshape the coral microbiome in situ without detectable off-target effects in the surrounding environment. *Commun Biol* **7**, 434 (2024).

104. C. R. Voolstra, J.-B. Raina, M. Dörr, A. Cárdenas, C. Pogoreutz, C. B. Silveira, A. R. Mohamed, D. G. Bourne, H. Luo, S. A. Amin, R. S. Peixoto, The coral microbiome in sickness, in health and in a changing world. *Nat. Rev. Microbiol.*, doi: 10.1038/s41579-024-01015-3 (2024).

105. M. J. H. van Oppen, J.-B. Raina, Coral holobiont research needs spatial analyses at the microbial scale. *Environ. Microbiol.* **25**, 179–183 (2023).

106. D. J. Hughes, J.-B. Raina, D. A. Nielsen, D. J. Suggett, M. Kühl, Disentangling compartment functions in sessile marine invertebrates. *Trends Ecol. Evol.* **37**, 740–748 (2022).