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RESEARCH

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High diversity of crustose coralline algae microbiomes across species and islands, and implications for coral recruits

Corentin Hochart¹, Héloïse Rouzé^{2,3}, Béatrice Rivière¹, Hans-Joachim Ruscheweyh⁴, Laetitia Hédouin², Xavier Pochon^{5,8}, Robert S. Steneck⁶, Julie Poulain⁷, Caroline Belser⁷, Maggy M. Nugues² and Pierre E. Galand^{1*}

Abstract

Background Crustose Coralline Algae (CCA) play a crucial role in coral reef ecosystems, contributing significantly to reef formation and serving as substrates for coral recruitment. The microbiome associated with CCAs may promote coral recruitment, yet these microbial communities remain largely understudied. This study investigates the microbial communities associated with a large number of different CCA species across six different islands of French Polynesia, and assess their potential influence on the microbiome of coral recruits.

Results Our findings reveal that CCA harbor a large diversity of bacteria that had not been reported until now. The composition of these microbial communities was influenced by geographic location, and was also closely linked to the host species, identified at a fine taxonomic unit using the 16S rRNA gene of the CCA chloroplast. We demonstrate the usefulness of these ecologically meaningful units that we call CCA chlorotypes. Additionally, we observed a correlation between host phylogeny and microbiome composition (phylosymbiosis) in two CCA species. Contrary to expectations, the CCA microbiome did not act as a microbial reservoir for coral recruits. However, the microbial community of coral recruits varied according to the substrate on which they grew.

Conclusions The study significantly expands the number of characterized CCA microbiomes, and provides new insight into the extensive diversity of these microbial communities. We show distinct microbiomes between and within CCA species, characterized by specific chloroplast 16S rRNA gene sequences. We term these distinct groups “chlorotypes”, and demonstrate their utility to differentiate CCA. We also show that only few bacterial taxa were shared between CCA and coral recruits growing in contact with them. Nevertheless, we observed that the microbial community of coral recruits varied depending on the substrate they grew on. We conclude that CCA and their associated bacteria influence the microbiome composition of the coral recruits.

Keywords Microbiome, Crustose coralline algae, Coral, Bacteria, Interaction, Coral recruitment

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Background

Crustose coralline algae (CCA) are calcifying red algae (Rhodophyta) that are widespread in coastal marine habitats across the globe [82]. In tropical waters, CCA are a key component of the ecosystem as they play a critical role in the construction and maintenance of coral reefs [10, 18, 51]. CCA also act as a natural CO₂ sink through photosynthesis and calcium carbonate production [82]. Another important ecological role of CCA is to induce the settlement and metamorphosis of various marine invertebrate larvae [14, 34–36, 39], including the ones of scleractinian corals [32, 64]. Coral larvae can show preferences for specific species of CCA as settlement substrate [29, 37, 65]. The algal chemical compounds [29, 80], and surface microbial biofilms [50, 74, 80] could both represent important coral settlement cues.

The microbial communities of CCA are thought to play an important role in scleractinian coral larval settlement [50, 80, 73, 74, 92]. Studies have shown that coral larvae exposed to specific bacteria and biofilms isolated from CCA can undergo metamorphosis and/or settling [31, 50, 58, 71, 80, 85]. Notably, bacteria isolates associated with the genus *Pseudoalteromonas* could be effective settlement inducers [50, 58, 74, 80, 81]. The inducing mechanism could be linked to secondary metabolites produced by the bacteria [57, 74]. In addition, the bacterial communities associated with the settlement substrata, and possibly the CCA, may act as a reservoir for the microbiomes of coral recruits, likely playing a critical role during coral ontogeny [73]. Indeed, some studies suggest that the environment surrounding the recruit is the main factor shaping the microbiome composition in the early stages of the coral's life [23, 40, 43]. However, although studies have been carried out separately on the microbial community of CCA and coral recruits, in situ studies including both groups of organisms, in various environments, and exploring their similarities are still lacking.

It is estimated that there are over 1,600 CCA species worldwide [47, 55], but despite their potential importance for coral larval recruitment, the few molecular studies that described their microbial diversity have focused mostly on three genera: *Porolithon*, *Neogoniolithon* and *Hydrolithon* [8, 27, 37, 38, 46, 50, 62, 63, 72, 73, 84, 90, 92]. These studies have shown that CCA bacterial communities are species-specific, with varying degrees of similarity among them. Additionally, community diversity varies across different CCA species [13, 16, 37, 73]. Although the CCA microbial community appeared to be relatively stable within species, studies have shown a shift in the microbial composition and diversity under environmental stressors, e.g. thermal variation, or when the algae are diseased [46, 62, 84, 92].

To date, the majority of studies exploring the diversity and role of CCA's microbiomes have focused on

two species: *Porolithon onkodes* [37, 50, 63, 70, 92] and *Neogoniolithon fosliei* [37, 70, 73, 86]. *P. onkodes* is an important reef building coralline alga, and is abundant throughout the Indo-Pacific. It has been shown to induce coral larval metamorphosis and settlement for different coral species [37, 89], but not necessary on its surface [37]. Its bacterial community is dominated by *Proteobacteria*, and often *Alphaproteobacteria* [37, 63, 70, 92]. *N. fosliei* is common in various parts of the tropical to subtropical Indo-Pacific and has also been reported to induce coral larval metamorphosis and settlement [29, 37, 79, 80], but at lower rate than *P. onkodes* [37]. The bacterial community of *N. fosliei* is dominated by *Alphaproteobacteria* followed by *Bacteroidetes* [70, 86], and *Gammaproteobacteria* in stress or aquaria conditions [37, 86]. Because the microbiome of only a few CCA species have been studied, the overall CCA microbial diversity remains largely underexplored.

The main goal of the study was to investigate the diversity and composition of the bacterial communities within a wide range of CCA species. This encompass both common and well-studied species, as well as various morphotypes of unknown cryptic CCA species, across distinct geographic locations in French Polynesia (Pacific Ocean). We collected as many different CCA morphotypes as possible from six islands of the Tuamotu archipelago, and sequenced the 16S rRNA gene to describe the bacterial communities, and to use the CCA chloroplasts to classify CCA morphotypes. We also sampled coral recruits growing on CCA, or turf algae, to compare their microbial communities with those of the algae they grew on, with the aim of testing whether the interaction could influence the coral's microbial composition.

Methods

Sample collection and environmental parameters

Crustose Coralline Algae (CCA), turf algae, and Pocillopora coral recruits were sampled by scuba diving in the Tuamotu archipelago, French Polynesia, Pacific Ocean. This sampling occurred across two to three different fore-reef sites at six islands (Faaite, Tahanea, Katiu, Makemo, Marutea and Hikueru) visited during the Tara Pacific expedition. The collection spanned from October 9 to 15, 2016, resulting in a total of 439 samples. The sampling design comprised three approaches:

1. The same four CCA species, *Neogoniolithon fosliei*, *Neogoniolithon frutescens*, *Porolithon gardineri*, and *Porolithon onkodes* were systematically sampled at the islands Faaite, Tahanea, Katiu and Makemo. They were chosen because they are common and easy to morphologically identify in situ and again at the surface. A total of three to five replicates per species were taken at each of the three sites visited at each

island. In total, 216 samples were gathered under this sampling design.

2. Sampling targeted all the CCA that had different morphotypes at two islands (Marutea and Hikueru). Three to five replicates were collected per morphotype. These CCA could not be clearly identified taxonomically and are further named collectively as “unknown CCA”. This dataset contained 53 samples.
3. Sampling targeted (i) the CCA *P. onkodes* associated or not to *Pocillopora* coral recruits, (ii) turf algae associated to coral recruits, (iii) coral recruits growing on/near CCA or turf algae, and (iv) adult *Pocillopora* colonies (Fig. 1). Turf algae represent a mix of species of diminutive algae with a canopy height < 1 cm. Coral recruits were considered ≤ 25 mm in maximum diameter. Since some *Pocillopora* species may be hard to distinguish, we use the genus level in this study. For this sampling design, we collected a total of 170 samples.

All samples were collected using a hammer and a chisel. All samples were gathered in individual plastic bags underwater. Once on board, one piece of each CCA was used for precise species identification under microscope, and another piece was stored in 1 mL of DNA shield and stored at -20°C for further microbiome analysis. Turf algae and corals were also stored in DNA shield at -20°C .

Environmental parameter of seawater temperature and salinity were measured using a CTD. In addition seawater samples for estimation of chlorophyll *a* and nutrient concentrations were collected and analysed following the Tara Pacific protocols [44].

DNA extraction and metabarcoding

Samples were disrupted mechanically in tubes containing a garnet matrix (Lysing Matrix A, MP, Biomedical, Santa Ana, CA, United States) with a FastPrep Instrument (MP, Biomedical) under the following conditions: speed: 6.0 m/s, time: 30s, pause time: 60 s, cycles: 3. Then, 500 μL of homogenized suspension was lysed with 50 μL of lysozyme (10 mg/mL), 3 μL of mutanolysine (50 KU/ml) and 3 μL of lysostaphine (4 KU/mL). After 1 h incubation at 37°C , 50 μL of Digestion Buffer and 25 μL of Proteinase K (20 mg/ml) were added, and a second incubation was performed for 30 min at 55°C . Then DNA extractions were conducted with the commercial Quick-DNA/RNA Kit (Zymo Research, Irvine, CA, USA). The V4-V5 region of the 16S rDNA gene was amplified by PCR with the universal 515 F-Y / 926R primers [54]. Since these primers also amplify eukaryotic 18 S rRNA genes, a nested PCR approach was applied with first a full-length amplification (20 cycles) using the 16S rRNA primers 27 F / 1492R [2] in order to increase

the target prokaryotic DNA, and a second amplification (25 cycles) using the 515 F-Y / 926R primers. The detailed PCR protocols are presented in Belser et al. [9]. PCR products were quantified and pooled after amplification and cleaned using AMPure XP beads. Libraries were prepared using the NEBNext DNA Modules Products (New England Biolabs, MA, USA) and NextFlex DNA barcodes (BIO Scientific Corporation, Austin, TX, USA) with 100 ng of purified PCR product as input. Libraries were subjected to Illumina sequencing at the Genoscope (Evry, France). All sequencing files were submitted to the European Nucleotide Archive (ENA) at the EMBL European Bioinformatics Institute (EMBL-EBI) under the project PRJEB74197. Sample provenance and environmental context are available on Zenodo [56].

Bacteria 16S rRNA sequence analysis

Adapters were removed with cutadapt (version 2.8) and an Amplicon Sequence Variants (ASVs) abundant table was built with DADA2 (version 1.14) as detailed in the scripts published in Zenodo [67]. The taxonomic annotation was performed using IDTAXA [49] with the training set corresponding to the SILVA database (v.138) [61] and a confidence threshold of 40. Eukaryotic ASVs (chloroplast and mitochondria) were identified based on taxonomic annotation following the criteria published in Zenodo [67] and removed from the dataset prior to bacterial analysis. In addition, bacterial sequences annotated at the family level as Comamonadaceae, Cutibacterium, Oxalobacteraceae, and Yersiniaceae were identified as reagent contaminants and removed from the dataset. The ASV abundance table is available on Zenodo (The ASV table is available on Zenodo (<https://zenodo.org/records/11352849>)).

CCA chloroplast 16S rRNA genes and distance tree building

Sequences automatically annotated as chloroplast with IDTAXA were manually checked against the nucleotide collection (nt) using the BLAST program [15], and only ASVs with a hit to chloroplast sequences on the ten best match were kept. Then, for each sample, the most abundant chloroplast ASV was used to characterize the CCA diversity. CCA samples that did not contain chloroplast ASVs were annotated as “undefined” ($n=2$), and CCA harboring several abundant chloroplast ASVs were annotated as “mixed” ($n=2$). CCA clades identified by using chloroplast sequences were named “chlorotypes”. A chlorotype ASVs distance tree was constructed using 16S rRNA sequences. Sequences were aligned, and the alignment was refined with MUSCLE (v3.8.1551) [22]. A distance matrix was computed with the KIMURA model of transition and transversion rates, and the tree constructed with the Fitch-Margoliash algorithm based on least squares principle in PHYLIP (v3.698) [24].

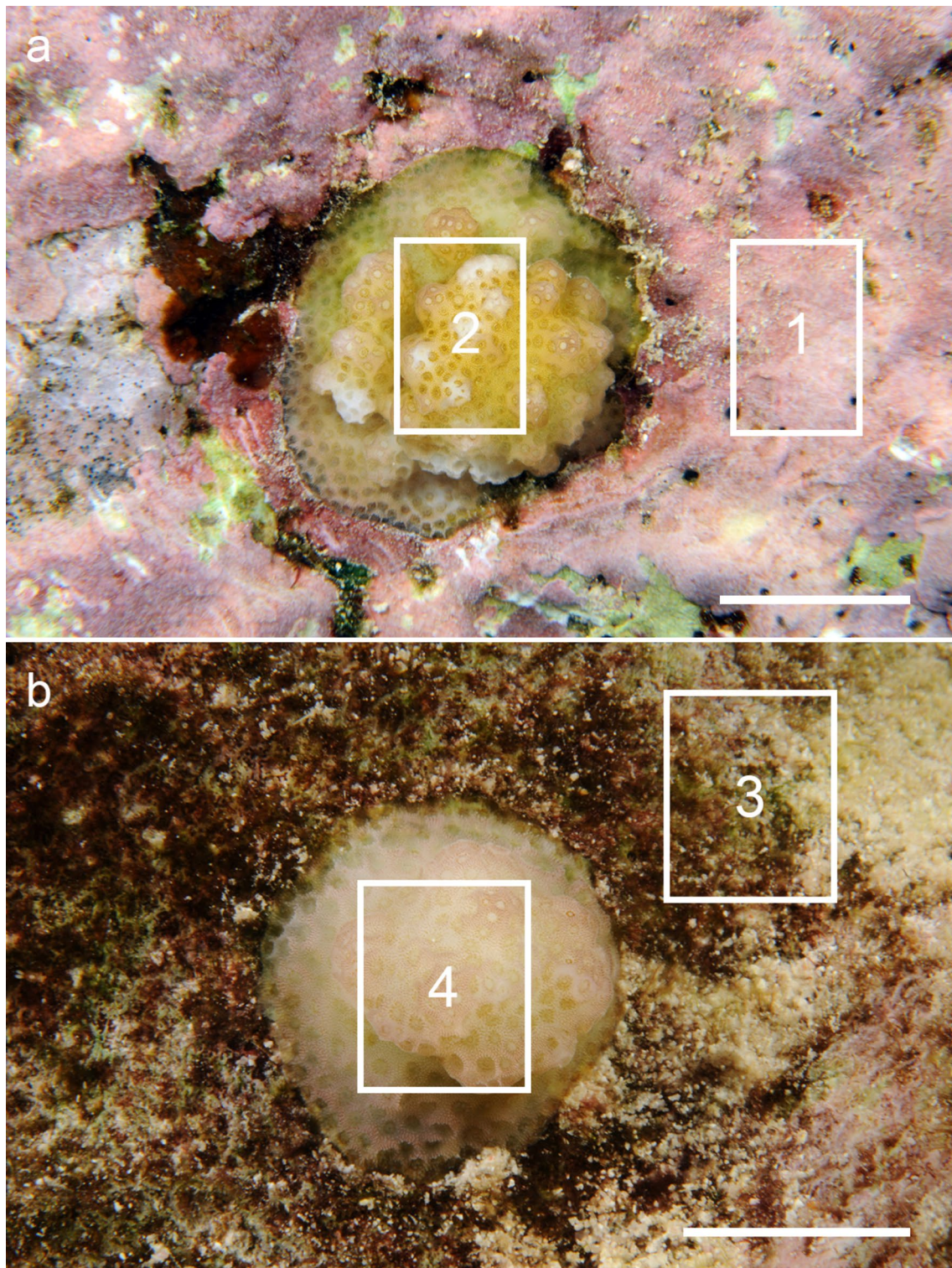


Fig. 1 Underwater photographs of **a** *Pocillopora* recruit in association with the CCA *P. onkodes*, and **b** *Pocillopora* recruit in association with turf algae. Two samples were taken for each association type. For the coral-CCA association: (1) the CCA associated with the recruit and (2) the recruit growing on/near the CCA. For the coral-turf association: (3) the turf associated with the recruits and (4) the recruit growing near the turf. Samples were also taken of adult *Pocillopora* corals and of *P. onkodes* not associated with corals (i.e., CCA- or turf-dominated area). The scale bar = 1 cm

Bacterial communities data analysis

The function `diverse` from “`diverse`” R package was used to compute the Shannon index after rarefying sample size to 15,000 sequences per sample with the function `rtk` from the R package “`rtk`”. Differences in α -diversity were tested with the Dunn’s test ($p < 0.01$) using “`Bonferroni`” as p-value adjustment, with the function `dunn_test` from “`rstatix`” R package. The NMDS ordination was performed with the function `metaMDS` from “`vegan`” package after computing Bray Curtis dissimilarities based on Hellinger transformed data. Beta-dispersion was computed with the function `betadisper` from “`vegan`” and the homogeneity of variance was test with TukeyHSD function from “`stat`” package. The average distance to the median (`adm`) for each tested group was obtained from `betadisper` results. Significant difference between groups were tested by PERMANOVA method with the function `adonis2` from “`vegan`”. For samples that were associated to each other (coral-algae microbiome association), we removed from the dataset the ASVs that were common to all sample types to avoid taking into account bacterial DNA that could have been transferred from one sample type to the other during sampling. To do so, we first identified the core ASVs of each group independently (coral, CCA, and turf algae) defined as having a relative abundance of more than $1e^{-4}$ percent and a sample prevalence of more than 50%. When a core ASVs from one group was detected in another group it was considered as potential cross-contamination and removed from that group’s dataset. We used a spearman based mantel test to verify if there was a correlation between the microbial community composition and the phylogeny of the CCA, or the environmental conditions, or the geography. The test was performed with the `mantel` function in “`vegan`”. For community composition distances were computed as Bray Curtis dissimilarities. For the CCA phylogeny, the phylogenetic distance was computed with PHYLIP (v3.698) [24] (see previous section). For the environmental conditions, Euclidian distances were computed using all available variables. The matrix of geographic distances between sampling points was computed based on their coordinates with `distm` in the “`geosphere`” package with the `haversine` method to account for the curvature of the Earth.

Results

Microbial community diversity across CCA, coral, and turf algae

In total, we captured 105,176 ASVs (108,595,985 sequence reads) from 439 samples across three different host types (266 crustose coralline algae (CCA), 90 corals and 30 turf algae) sampled across 6 islands (Fig. 2a).

Microbial community diversity (Shannon index) differed between the three host types (Fig. 2b, Dunn’s post

hoc, $p < 1e^{-07}$). Corals had the lowest microbial community diversity followed by the CCA, and finally turf algae had the highest. Within CCA, we did not observe any difference in community diversity between genera (Supplementary Fig. 1a) and species (Supplementary Fig. 1b), except between *P. onkodes* and *P. gardineri* (Dunn’s post hoc, $p < 5e^{-03}$).

Microbial community composition was significantly different between the three host types (Fig. 2c; PERMANOVA, $p < 1e^{-4}$; Supplementary Table 1), although there was no homogeneity of variance between groups. Community composition differed also between known CCA genera and species (*N. fosliei*, *N. frutescens*, *P. gardineri*, and *P. onkodes*; PERMANOVA, $p < 1e^{-4}$; Supplementary Table 2). Microbial communities associated to the two *Porolithon* species were less clearly separated on the nMDS ordination than the communities associated to the *Neogoniolithon* species (Fig. 2c). *Pocillopora* had highest variation in community composition (average distance to the median (`adm`): 0.60), followed by *Porolithon* (`adm`: 0.58), *Neogoniolithon* (`adm`: 0.56) and turf algae (`adm`: 0.52).

We did not detect any ASVs that were present in all samples across the different host types, neither within all CCA samples, nor within all coral samples (Supplementary Fig. 2). Conversely, for the turf algae, 81 ASVs were found to be present in all 30 samples. The ASV with the highest occurrence over all samples (`asv0000053`, 95% of the samples) belonged to the *Ruegeria* genus (*Rhodobacteraceae* family class *Alphaproteobacteria*). The two most abundant ASVs (`asv0000003` and `asv0000001`) belonged to the genus *Endozoicomonas* (family *Endozoicomonadaceae*). These two ASVs were most abundant in corals (28–32% of the sequences) than in CCA (<0.001%), and turf algae (<0.001%).

CCA chloroplast classification

For CCA, we isolated the 16S rRNA ASVs originating from eukaryotic chloroplasts, and identified a total of 55 different chloroplast ASVs that we called chlorotypes (Fig. 3). We were not able to define a chlorotype in four of the CCA samples. We constructed a phylogenetic tree based on 16S rRNA sequences of the chloroplasts. The chlorotype phylogeny showed that *N. fosliei* samples grouped together separated from the other CCA. Another cluster contained the second *Neogoniolithon* (*N. frutescens*), and then the 2 *Porolithon* spp., in addition to unknown CCA (Fig. 3). Within *Porolithon*, the 2 species did not separate clearly into distinct clades. *P. gardineri* chlorotypes were the more dispersed across the tree. Each chlorotype was observed in only one species, except for the unknown CCAs (Fig. 3). We used the tree to name some of the unknown species based on their chlorotype. If a chlorotype was shared between a unique known CCA

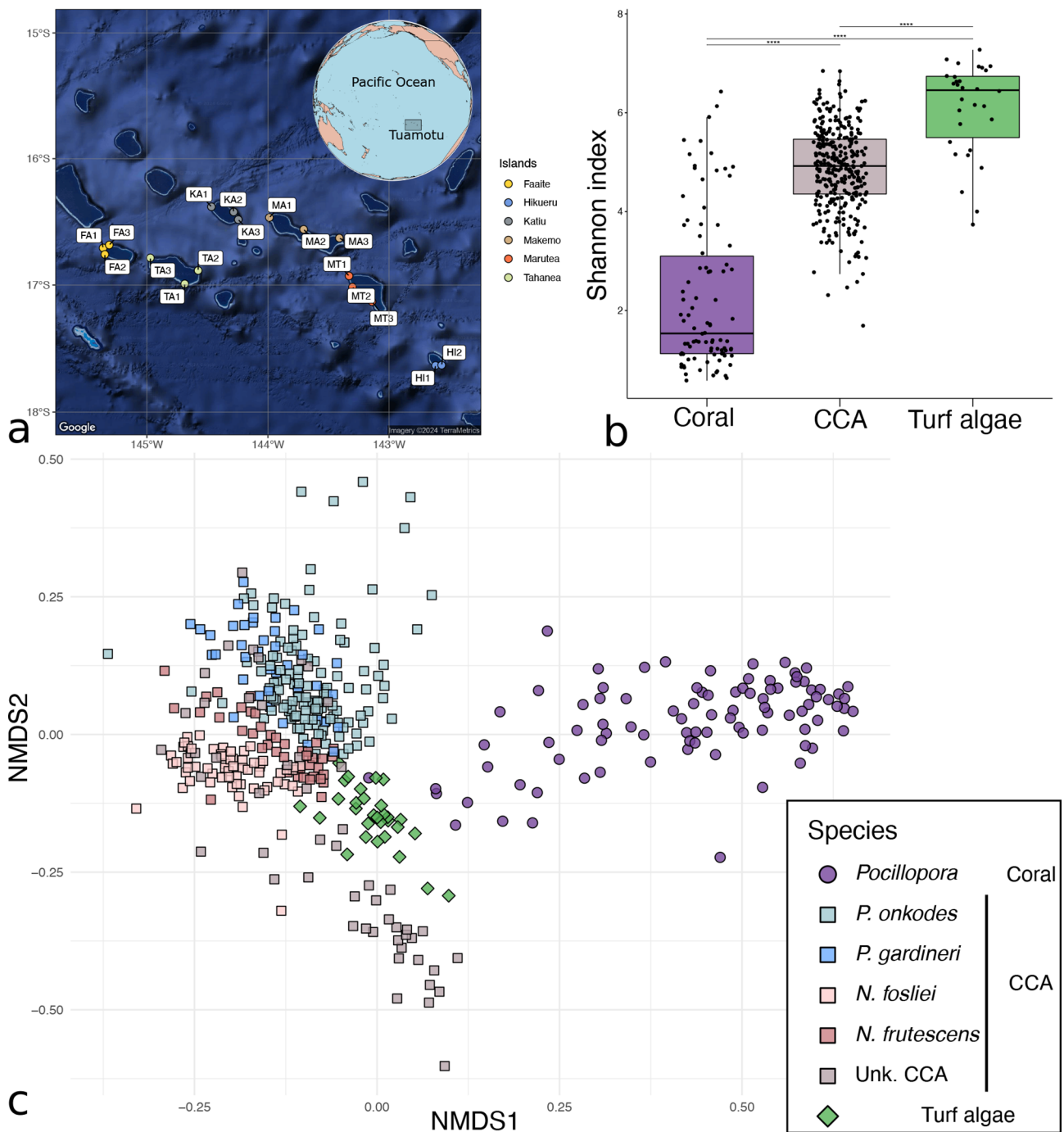


Fig. 2 Bacterial diversity and community composition of crustose coralline algae (CCA), coral, and turf algae across six islands in the Tuamotu archipelago (French Polynesia). **a** Map of the islands and sites sampled. **b** Microbial community diversity (Shannon index) in the different host types. The box plot horizontal bars show the median value, the box indicates the first and third QRs, and the whiskers indicate 1.5*IQR. The asterisks indicate p values < 0.001. **c** Non-metric multidimensional scaling (nMDS) of bacterial community composition in the corals (*Pocillopora*), CCA (*N. fosliei*, *N. frutescens*, *P. gardineri*, *P. onkodes*, and unk. - unknown), and turf algae (stress: 0.17)

species and unknown CCA, all unknown CCA bearing this chlorotype were assigned to this known species (e.g. all unknown CCA having chlorotype *asv0000274* were considered as *N. frutescens*). We could assign 25 of the unknown CCA based on the chlorotypes found in the

four known species. The other 27 samples were associated to ten chlorotypes that were not present among known CCA species.

P. onkodes samples were mainly associated to three chlorotypes, *asv0000043* being the most frequent (found

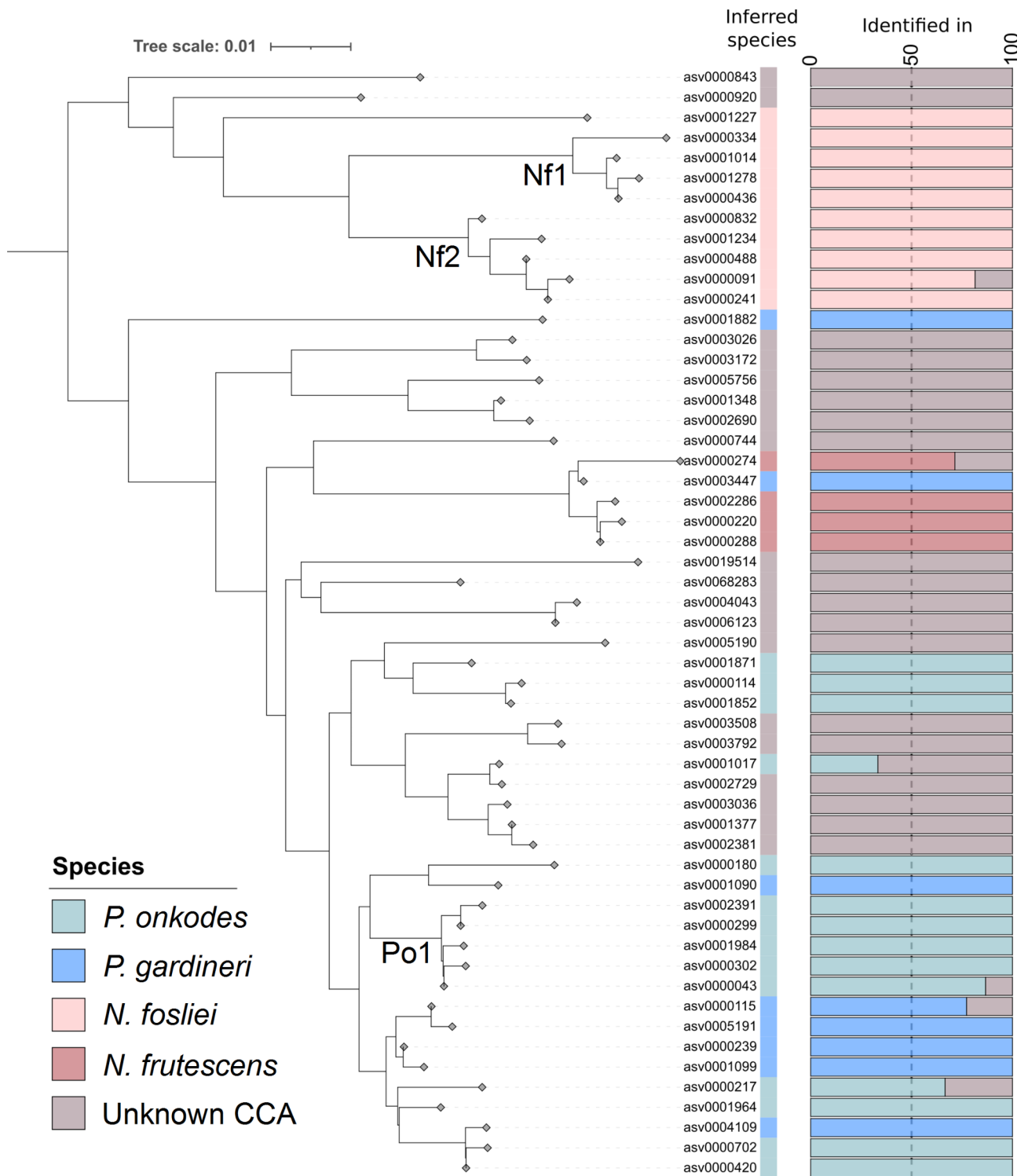


Fig. 3 Distance tree based on chloroplast 16S rRNA genes (chlorotype) from CCA samples across six islands of French Polynesia. The bar on the right shows in which sampled CCA species the chlorotype was found. The vertical ribbon indicates the species names inferred through the chlorotype. Nf1, Nf2 and Po1 are clades mentioned in the description of the CCA bacterial community composition

in $n=26$ samples), followed by two less common ones (*otu0000299*, $n=14$, and *otu0000420*, $n=8$). *P. gardineri* samples were dominated by the chlorotype *asv0000115* ($n=17$), followed by *asv0000239* ($n=13$). In *N. fosliei* samples, ten different chlorotypes were identified, with *asv0000091* present in 22 samples. For two *N. fosliei* samples, we observed a mixed of two or more chlorotypes. Interestingly, the occurrence of *N. fosliei* chlorotypes was significantly related to the geography (mantel, p -value <0.05). In *N. frutescens*, four chlorotypes were identified with three chlorotypes being the most frequent (*asv0000274*, $n=10$; *asv0000220*, $n=18$; *asv0000288*, $n=13$).

Microbial communities associated to CCA

A total of 58,572 prokaryotic ASVs were detected in the known and unknown CCAs species sampled across five islands. Among these ASVs, nearly 36% (21,207) could not be annotated at the genus level and around 28% (16,589) at the family level. The accumulation curve based on community richness shows that diversity increased with the addition of samples from different CCA species (Fig. 4a). Starting with *P. gardineri*, we observed an increase followed by a plateau, followed by a sharp increase again when adding *P. onkodes* samples. Within *P. onkodes*, diversity increased again at each addition of new chlorotypes. The addition of the two *Neogoniolithon* species led to a slower increase in diversity. Finally, within the unknown CCAs, we observed sharp increases when adding new chlorotypes (Fig. 4a). We also compared alpha community diversity between islands. For the *Neogoniolithon* species, we did not observe any significant effect of the islands, but for *P. onkodes*, samples from Hikueru had significantly lower community diversity (Dunn's post hoc, $p<0.05$).

At the community level, there were significant differences in bacterial community composition between CCA genus, species, and chlorotypes (Fig. 4b; PERMANOVA, $p<1e^{-4}$; Supplementary Table 2). Unknown CCAs for which the species name was inferred from their chlorotypes grouped together with the known species that had the same chlorotype. Microbial community composition between chlorotypes was significantly more different for different species and genus than within the same species or genus (Supplementary Fig. 3a). Most of the chlorotypes from different species of *Neogoniolithon* had significant different community composition (Supplementary Fig. 3b). Community composition of different chlorotypes of the same species were mostly not significantly different (Supplementary Fig. 3c).

For each known species, geography had a significant effect on microbial community composition, except for *N. fosliei* (Supplementary Fig. 4). Environmental conditions (sea surface temperature, salinity, silanol,

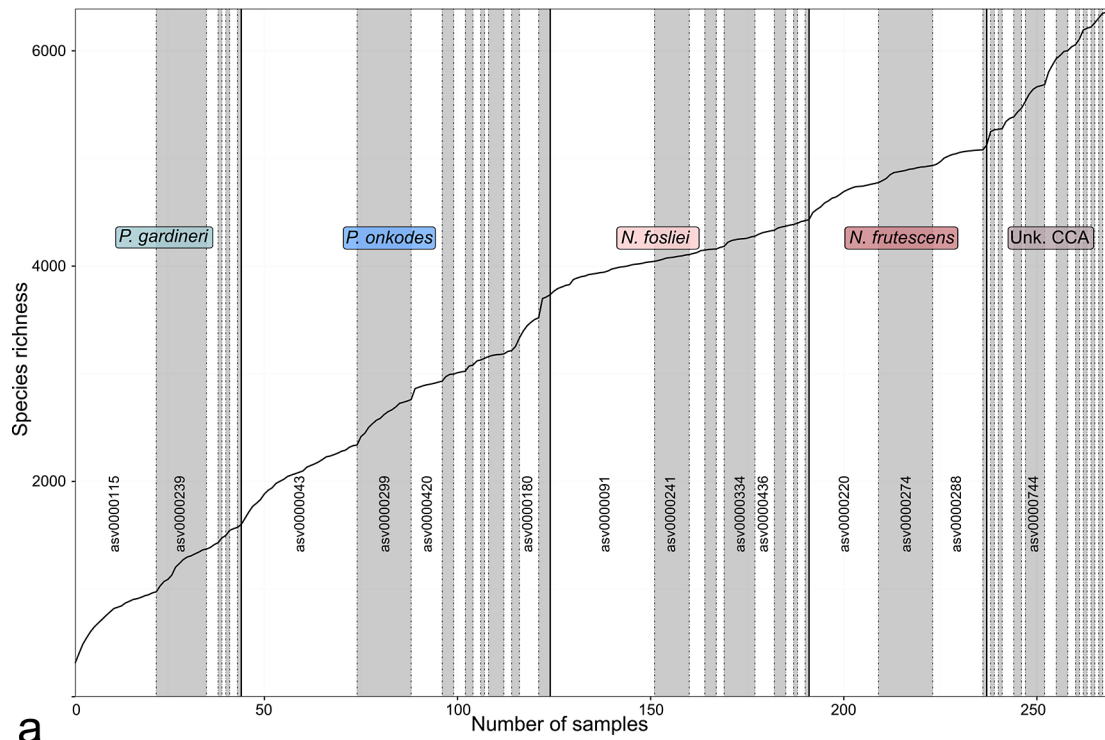
chlorophyll a concentration, pH, and phosphate concentration) had higher significance in *N. fosliei* and less in *P. gardineri*, and no correlation in *N. frutescens* and *P. onkodes*. The bacterial communities were significantly different between islands (PERMANOVA, $p<1e^{-4}$; Supplementary Table 3). We also used Mantel tests to assess putative phyllosymbiosis between the CCAs and their microbiomes. Phyllosymbiosis is defined by the evolutionary pattern in which the phylogeny of host organisms correlates with microbial community composition. There was a significant correlation between microbiome and chlorotypes for *P. onkodes*, *N. fosliei* and for the overall CCAs ($p<0.01$; Supplementary Fig. 4). However, we did not observe significant evidence for phyllosymbiosis at the chlorotype level in *P. gardineri* and *N. frutescens* ($p>0.01$; Supplementary Fig. 4).

In *P. onkodes*, most of the samples grouped together according to their chlorotype, indicating that different chlorotypes had different microbial community composition (Supplementary Fig. 5a, Supplementary Table 3). However, some CCA assigned to different chlorotypes harbored similar community composition. Interestingly, these chlorotypes belonged to the same monophyletic clade that we named Po1 (*asv0002391*, *asv0000299*, *asv0001984*, *asv0000302*, *asv0000043*; Fig. 3). These chlorotypes were found in different islands and sites, while the chlorotypes *asv0000180* and *asv0000420* were found only in the site 3 of Tahanea and the site 1 of Faaite, respectively.

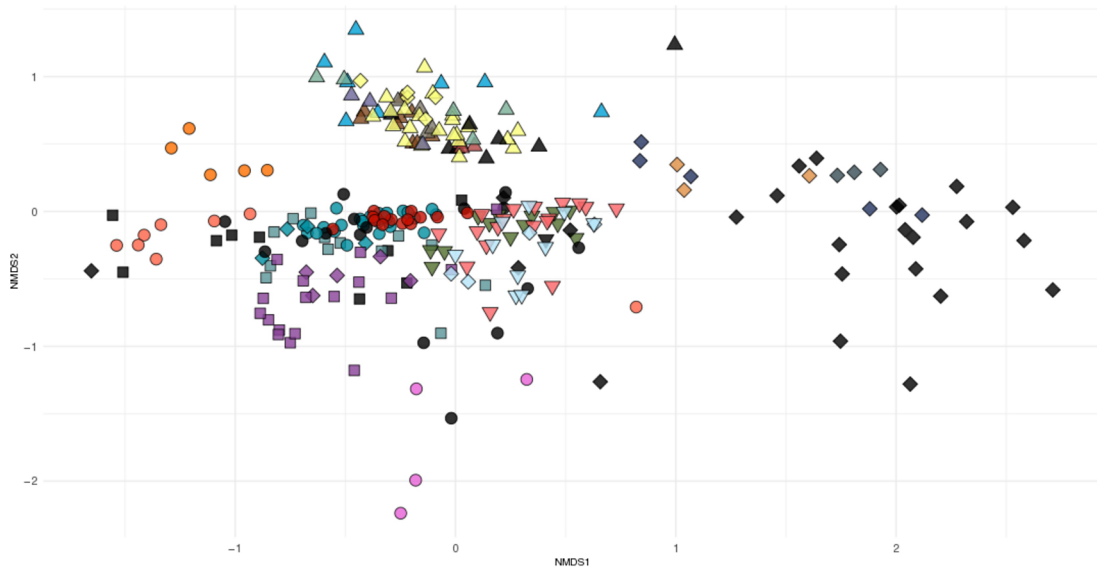
In *P. gardineri*, the samples grouped on the ordination plot by islands and by chlorotypes (Supplementary Fig. 5b, Supplementary Table 3). More specifically, most island sites were associated to one chlorotype only or chlorotypes phylogenetically close to each other. For instance, site 1 of Tahanea had the chlorotype *asv0000239*, while site 2 of Faaite had the neighbor chlorotypes *asv0000239* and *asv0001964* (Supplementary Fig. 5b) (Fig. 3).

In *N. fosliei* (Supplementary Fig. 5c), we observed two distinct group of samples that corresponded to two monophyletic clades of chlorotypes (one clade that we named Nf1 with *asv0000334*, *asv0001014*, *asv0001278*, *asv0000436*; another clade named Nf2 with *asv0001234*, *asv0000488*, *asv0000091*, *asv0000241*; Fig. 3). Additionally, the microbial community affiliated to the chlorotype *asv0001227*, which forms an external branch in the phylogenetic tree (Fig. 3), differed greatly from the two above mentioned clades. We did not observe any specific patterns between the chlorotypes for the islands and sites.

In *N. frutescens*, we did not observe any clear pattern in microbial community composition according to islands, sites or chlorotypes (Supplementary Fig. 5d). However, most of the island sites were associated with only one chlorotype. Finally, *N. frutescens* was the only species in



a



b

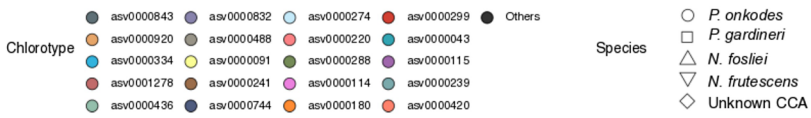


Fig. 4 Bacterial diversity and community composition of CCA in 6 islands of French Polynesia. **a** Accumulation curve of community richness. Vertical plain lines delimit the different CCA species; vertical dashed line delimit the different CCA chlorotypes. Chlorotypes found in more than eight samples have their names displayed. **b** Non-metric multidimensional scaling (nMDS) of bacterial community composition in known and unknown CCA (stress=0.18). Colors represent the different chlorotypes and shapes indicate the CCA species names

which chlorotypes did not explain differences in community composition. Interestingly, the mean phylogenetic distance between chlorotypes within *N. frutescens* was the lowest among known CCAs (Fig. 3).

At the order level, the CCA communities were overall dominated by *Flavobacteriales* (mean 17.2%). Other abundant groups included *Pirellulales* (10.8%), *Chitinophagales* (8.78%), *Alteromonadales* (8.09%), *Rhodobacteriales* (7.03%), and *Rhizobiales* (5.92%), with significant difference in abundance between CCA species (Dunn's test, $p < 0.01$). The unclassified ASVs at the genus level represent 15.3% of the sequences in the CCAs samples overall, and 9.4% at the family level. *Porolithon* had the lowest number of unknown bacteria, followed by the two *Neogoniolithon* species, and finally the unknown CCAs (23.1% of non-classified ASVs at the genus level).

N. fosliei had the highest proportion of ASVs unique to the species (282), followed by *P. onkododes* (196), *N. frutescens* (155) and *P. gardineri* (68), which represents respectively 41, 14, 24 and 13% of the overall microbial community of each species (Fig. 5). These unique ASVs were mostly affiliated to *Flavobacteriales* and *Alteromonadales*. The pool of unknown CCAs had 641 unique ASVs, which represented in average 19% of the sequences and were dominated by *Pirellulales*. Interestingly, the two *Porolithon* species shared 105 unique ASVs, which

represents $25 \pm 3\%$ of the overall sequences, while *Neogoniolithon* only shared 21, which represents $3 \pm 1\%$ of the sequences. We found 62 ASVs common to the four known CCA species, and 41 ASVs common to all species, which represent respectively $9 \pm 4\%$ and $10 \pm 4\%$ of the sequences of these species. They were mostly affiliated to *Rhodobacterales*, *Rhizobiales*, *Cyanobacteriales*, *Phycisphaerales*, *Pirellulales* and *Caulobacteriales* (Fig. 5).

Microbial communities of corals associated with CCA vs. turf algae

The dataset was composed of 90 *Pocillopora* coral samples of different sizes, among which 30 associated with the CCA *P. onkododes*, 30 associated with turf algae, and 30 not associated to any algae. It also contains 50 *P. onkododes* CCA samples, among which the 30 above-mentioned associated with *Pocillopora*, and 20 not associated to any other macro-organisms. Finally there were 30 turf algae samples associated with the above-mentioned *Pocillopora*.

The diversity of microbial communities of *Pocillopora* recruits associated with the CCA and turf algae were similar to each other, and higher than the diversity of unassociated adult *Pocillopora* communities (Fig. 6a) (Shannon index, Dunn's post hoc, $p < 0.001$) (Supplementary Table 4). Inversely, the diversity of the CCA *P.*

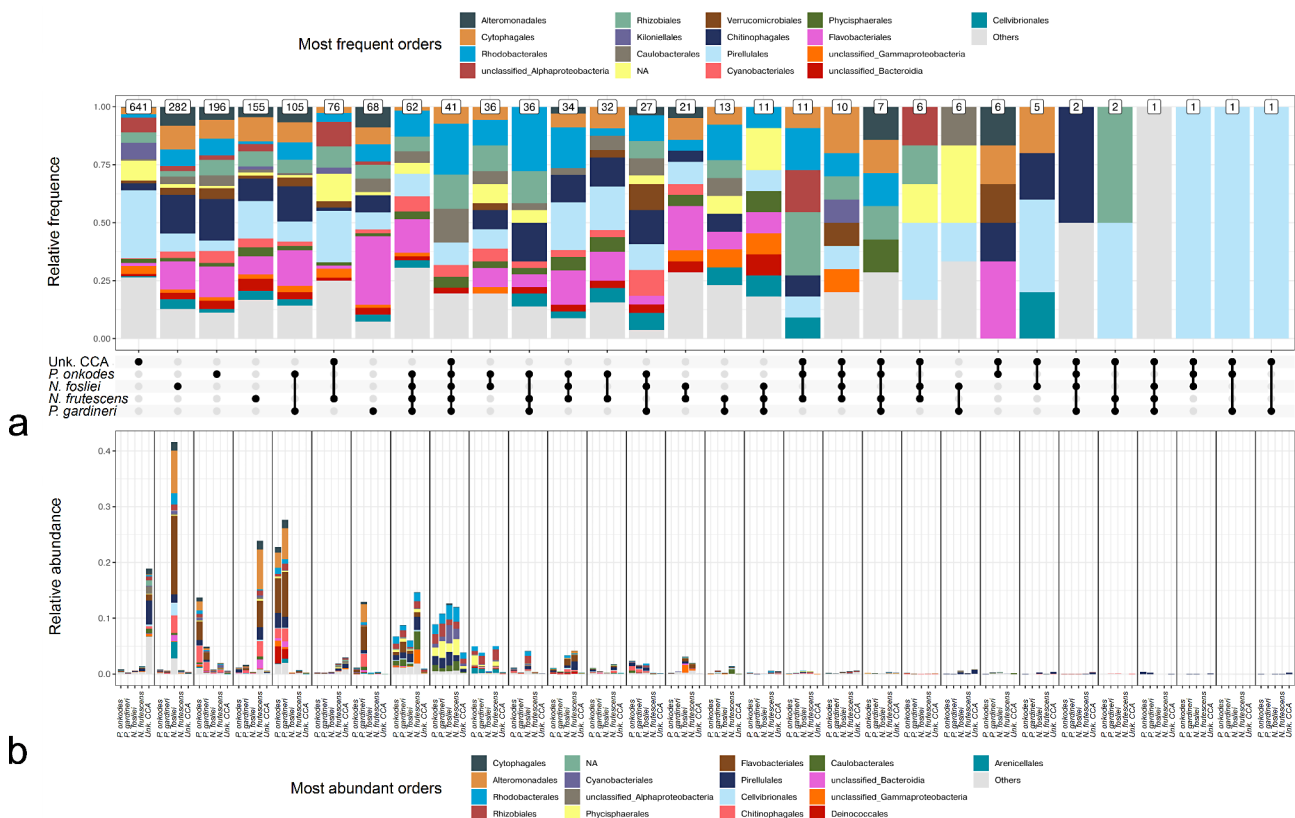


Fig. 5 UpSet plot showing the number (a) and abundance (b) of ASVs unique to or shared between CCA species

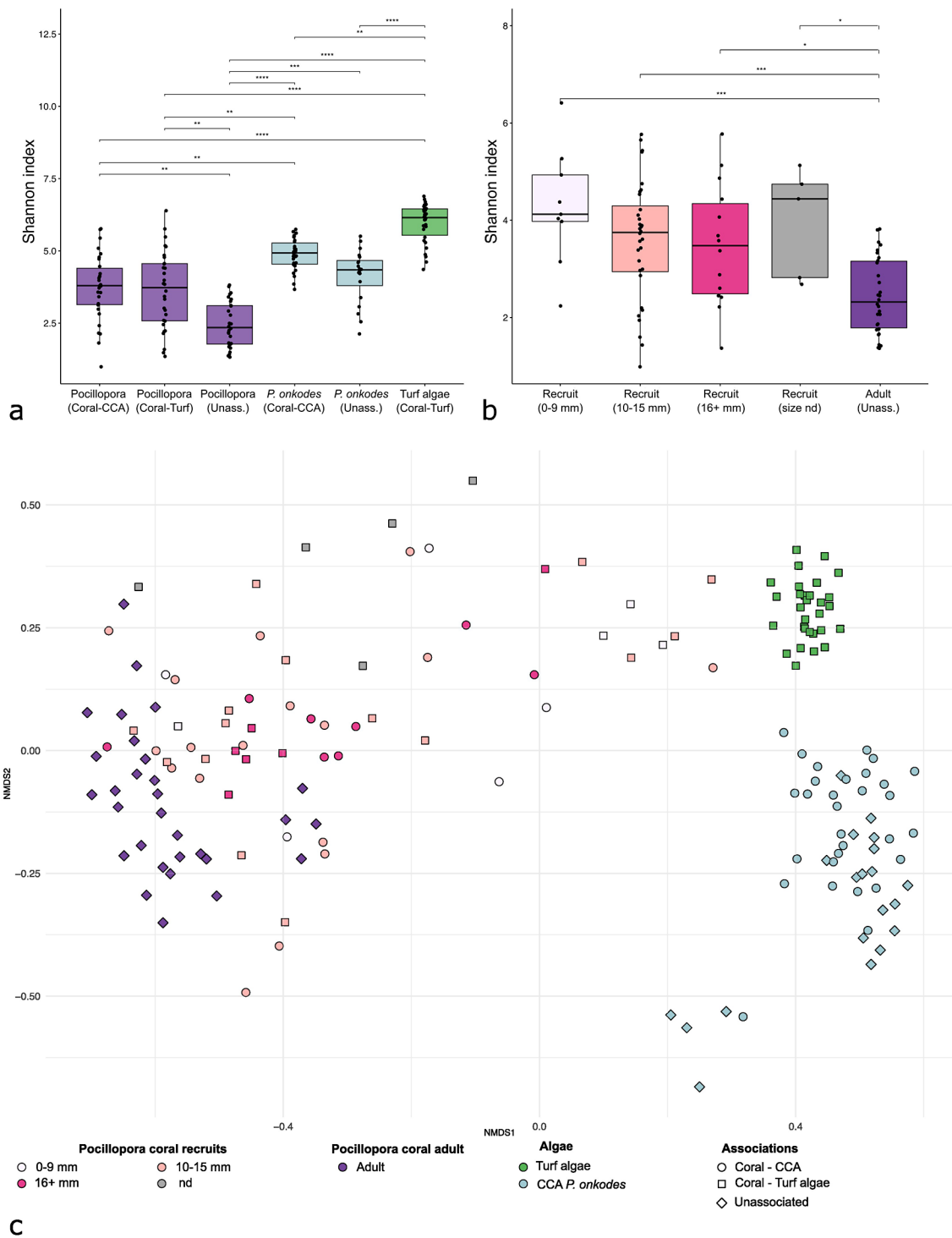


Fig. 6 Bacterial diversity and community composition of the coral *Pocillopora*, the CCA *P. onkodes* and turf algae. **a** Shannon diversity for the coral *Pocillopora* associated or not to a CCA or turf algae, for the CCA *P. onkodes* associated or not to the coral, and for the turf algae associated with the coral. **b** Shannon diversity in different colony size of *Pocillopora*. **c** Bray-Curtis based nMDS ordination (stress=0.15) of the microbial community composition coral recruits, adult corals, turf algae, and CCA *P. onkodes* under different association conditions. nd: not determined; Unass.: unassociated

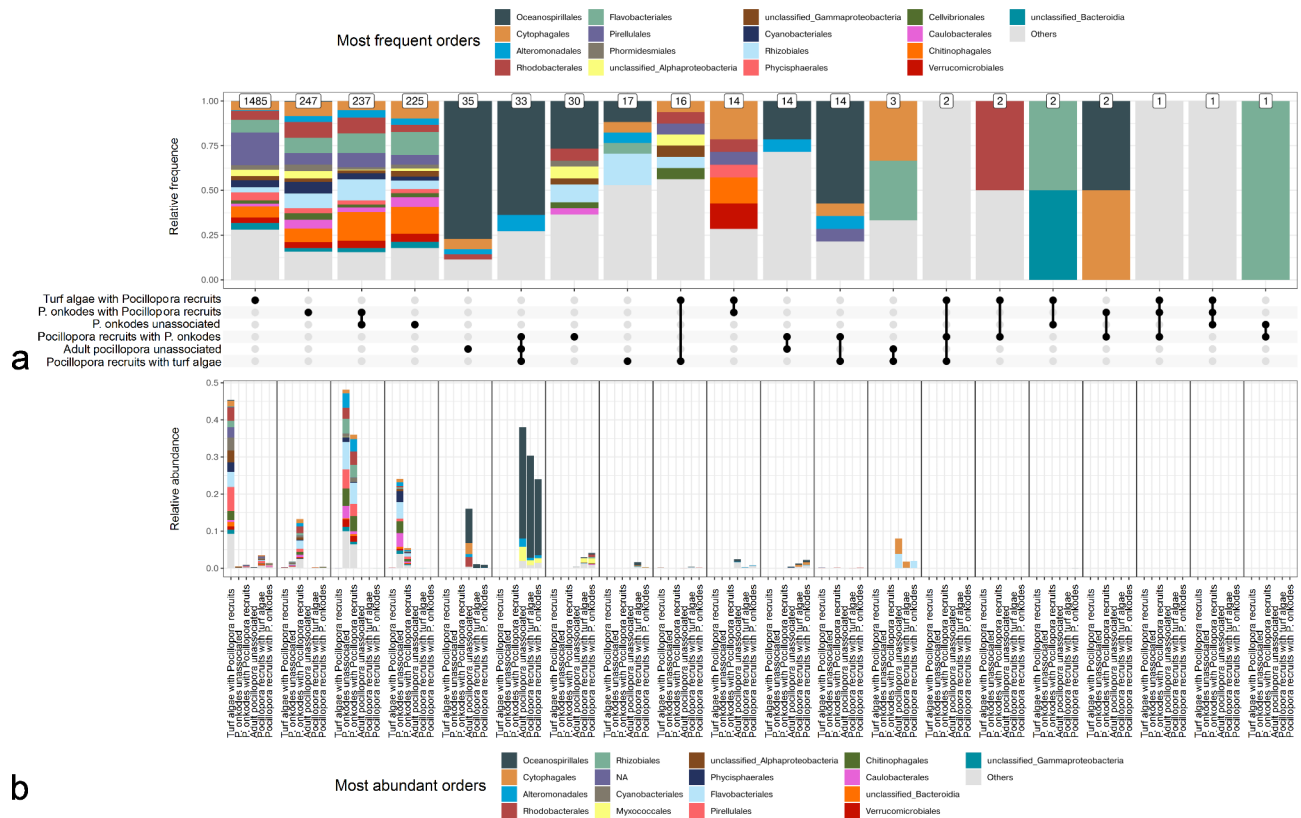


Fig. 7 UpSet plot showing the number (a) and abundance (b) of ASVs unique or shared between *Pocillopora* (recruits and adult), *P. onkodes* and turf algae microbiome

onkodes communities associated or not were similar to each other. Turf algae microbial communities had highest diversity. More specifically for *Pocillopora* corals, recruits (<20 mm in size) had a more diverse bacterial communities than unassociated adult colonies (Fig. 6b) (Shannon index, Dunn’s post hoc, $p < 0.001$).

At the community level, for *Pocillopora*, there were significant differences in composition between the bacterial communities that were associated to an algae and the ones that were not (PERMANOVA, $p < 0.001$), and overall, bacterial communities were significantly different depending of the association types (unassociated, coral-CCA and coral-turf algae) (Fig. 6c) (PERMANOVA, $p < 0.005$) (Supplementary Table 5). *Pocillopora* communities were thus variable in their microbial community composition while turf algae, and then *P. onkodes*, were more homogenous (Fig. 6c) Microbial community composition also differed significantly between adult corals and recruits (PERMANOVA, $p < 0.05$).

For turf algae, we observed a difference in community composition between the two islands Marutea and Hikueru, and Tahena, while for the coral *Pocillopora* and the CCA *P. onkodes*, the communities were different between the three islands (PERMANOVA, $p < 0.001$). Additionally, coral recruit communities were significantly

different between islands (PERMANOVA, $p < 0.001$) and association types (PERMANOVA, $p < 0.015$). However, the interaction between islands and association types were tested significant (PERMANOVA, $p < 0.05$). Tested by island, the coral recruit communities were not significantly different according to the associated algae.

We did not find any ASVs common to all samples (Fig. 7). In total, we observed only 24 ASVs shared between the coral *Pocillopora* and algae, which accounted for less than 5% of the sequences. Sixteen of these ASVs were shared between the sample groups *Pocillopora* associated with turf algae, and turf algae associated with *Pocillopora*. They belonged mostly to diverse *Alphaproteobacteria* and *Gammaproteobacteria*. Only four ASVs belonging to *Oceanospirillales*, *Cytophagales* and *Flavobacteriales* were found to be common between *Pocillopora* and the CCA *P. onkodes*. Few ASVs were found to be specific to *Pocillopora* ($n = 146$), belonging mostly to the *Endozoicomonadacea* family, but they accounted for 34 to 66% of the overall communities.

The *Endozoicomonas* genera (37.6%) (order: *Oceanospirillales*) dominated the *Pocillopora* microbiome (asv000001 and asv000003). Then followed the *Flavobacteriales* (7.02%), *Cytophagales* (6.37%), *Myxococcales* (3.52%) and *Alteromonadales* (3.03%). The algae

were dominated by *Flavobacteriales* (Turf: 9.53%; *P. onk*: 15.6%) and *Pirelluales* (Turf: 12.2%; *P. onk*: 7.98%), as well as *Chitinophagales* (12.2%) for *P. onkodes*. *Pocillopora* recruits had significantly less *Oceanospirillales*, especially *Endozoicomonas*, compared to the adult *Pocillopora* community. While the *P. onkodes* associated community had significantly less unclassified *Gammaproteobacteria*.

Turf algae were characterized by a high number of unique ASVs, twice as many as in *P. onkodes*, and ten times as many as in *Pocillopora* (Fig. 7). These ASVs accounted for 45% of the total abundance of the turf algae community. *P. onkodes* associated to coral recruits and unassociated harbor respectively 247 and 225 unique ASVs, which represent 24 and 13% of the total community. Additionally, *P. onkodes* associated to coral recruits and unassociated shared 237 common ASVs, accounting for $42\% \pm 9\%$ of the overall community of the CCA.

Discussion

The diversity of CCA microbiomes

This study considerably increased the number of CCA analysed to date with up to 55 different chlorotypes detected. However, we could not saturate the discovery curve of the microbial community diversity since each addition of a new CCA species, or chlorotype, to the study increased the microbial diversity. It shows that there is still a large potential of undescribed microbial communities among the >1,600 CCA species known globally [55]. This newly described diversity adds to the diverse communities of microorganisms recently reported in corals, tropical fish, and reef plankton [17, 26], and demonstrates that reef ecosystems harbor a tremendous undescribed microbial diversity.

Our study shows that chlorotypes (based on chloroplast 16S rRNA) could be used as a useful barcoding tool to differentiate genus of CCA, and even intraspecific variants. Classical CCA identification requires examination of different vegetative and reproductive features [20]. However, due to the need of expert eyes, and laboratory microscopy processing, the identification and differentiation of taxa based on morphological and development features remain challenging [20], especially since geographic, seasonal and environmental conditions can influence morphology [45, 76, 91]. To elucidate the phylogeny of CCA, molecular approaches have been increasingly applied, mainly using specific markers such as the 18 S rRNA gene, the chloroplast *PsbA* gene, and the mitochondrial *cox1* gene [5–7, 83]. We were not able to directly compare the 16S chlorotype from this study to the ones from other known CCA species due to lack of reference sequences for this gene. Our chloroplast 16S rRNA distance tree was nevertheless precise enough to separate groups of CCA, to explain that variations in microbial composition within a same CCA species

followed chlorotype delineations, and that the addition of chlorotypes to the rarefaction curve added microbial diversity. It suggests that chlorotypes could in some cases represent taxonomic units that are not distinguishable by classical identification techniques. The potential for chlorotypes to resolve finer clades varied between species giving for instance better results for *P. onkodes* than for *N. frutescens*. However, the chlorotypes did not allow a precise phylogenetic separation of monophyletic groups, and did not directly support the known phylogenetic separation of CCA in the tree. For instance, the two *Porolithon* species did not form a monophyletic clade.

The CCA microbiome communities were significantly different between CCA genera, but there were also significant differences between species from the same genus. It had been observed earlier for *Porolithon* [70], and our finding thus extends these observation to *Neogoniolithon*. Previous studies have also shown CCA species specific microbiomes [37, 73], but our results go beyond current knowledge by showing that there were often differences in microbial community composition between CCA chlorotypes. We showed that even at the fine chlorotype or sub-species level, microbial communities were specific and strongly linked to the host genetics. It provides solid evidence of the specificity of the CCA – microbiome association.

We originally hypothesized that CCA would show patterns of phyllosymbiosis as previously observed in corals [60]. Our results partly confirmed this hypothesis, as we observed phyllosymbiosis in *P. onkodes* and *N. fosliei*. In these cases, the host chlorotype was the main determinant of microbiome composition before the environment, and the geographic location. Phyllosymbiosis patterns may emerge as a consequence of different factors like co-diversification, host and microbial biogeography interaction, or host trait preference [12, 53]. Further work should be conducted by integrating sets of phylogenetic markers (SSU, LSU, psbA, COI, 23 S) [66], as well morphological taxonomy, to confirm our findings.

In our study, CCA were overall dominated by *Flavobacteriia* (phylum: *Bacteroidota*) followed by *Alphaproteobacteria* (phylum: *Proteobacteria*). These two phyla were also identified as co-dominants in several other CCA microbiomes studies: in *P. onkodes* collected from the China Sea [92], *Neogoniolithon sp.* from the Mediterranean sea [27], and in *N. mamillare* from the Caribbean sea [46]. However, this is not always the case as seen with the absence or rarity of *Bacteroidota* in *N. brassica-florida* from Mediterranean sea [62]d *fosliei* and *P. onkodes* from Moorea, French Polynesia [37]. The differences may be due to different sampling design as some analyses were conducted on CCA from aquarium a day after the collection from the back reef [37], and aquaria and field microbial communities of *Neogoniolithon sp.* have been

shown to differ significantly [27]. Additionally, they may be spatial or seasonal variations in communities as seen for several other benthic groups (e.g. corals, sponges) (White et al., [21, 88]. For instance, *N. fosliei* microbial community changed from *Alphaproteobacteria* to *Bacteroidota* (formerly *Bacteroidetes*) under rising sea surface temperature [86]. This change in the microbiome composition was accompanied by a reduction in the ability of *N. fosliei* to induce coral larval metamorphosis of *Acropora millepora* by half [86]. Similarly, our CCA were sampled following the 2016 bleaching event which increased heat stress up to 9.2 °C weeks and caused large-scale coral mortality in the Tuamotu Archipelago [30].

In our study, the most frequent CCA ASV (asv0000053) was identified as *Ruegeria profundii* [93] (100% identity). Interestingly, bacteria belonging to the *Ruegeria* genus could have antimicrobial activities, and notably against the diverse marine host-pathogen genus *Vibrio* (family *Vibrionaceae*) [48]. *Ruegeria* are also known for their ability to form biofilms and their inhibition capabilities [19, 77]. Additionally, a strain similar to the one we found (*Ruegeria mobilis*, 99.3% identity), isolated from seawater in Eilat (northern Red Sea, Israel), was beneficial for larval development in the octocoral *Rhytisma fulvum* [25]. However, members of this bacterial genus were also found associated with diseased corals [3, 78]é et al., [68]), so its role remains to be precisely determined. We also noted that asv0003145 represented a bacterium assigned to another genus from the *Roseobacter* family, *Roseovarius*, which contains the known pathogen *Roseovarius crassostreae*, the causative agent associated with juvenile oyster disease [11].

Microbial communities shared between CCA and the coral *Pocillopora*

In our study, some bacterial taxa were shared between CCA and corals. This observation is interesting as microbial communities have been hypothesized to play a role in coral larvae recruitment and recruit health, but also as reservoir for the development of the coral's microbiome [73]. However, here, all the bacterial taxa shared between the CCA and the associated coral recruits were found in very low abundance. Although limited to *Pocillopora* corals, these results suggest, contrary to an earlier hypothesis [73], that only few bacteria could have been transferred from the algal substrata to the coral colony. Furthermore, we observed that there were fewer shared taxa in adult corals compared to recruits. It implies that although bacteria taxa from the environment, here the algal substrate, may settle on coral recruits, further coral-bacteria, and bacteria-bacteria interactions select a stable and less diverse bacteria community in adult corals. Further studies on younger coral recruits are needed

to explore the potential interaction between the algal microbiome and that of coral recruits.

Although there was no clear transfer of bacteria from CCA to corals, we noted that the composition of the coral recruit microbiome differed according to their surrounding substrate. The coral microbial community may thus be influenced by the substrate. One explanation maybe the release of algal-specific metabolites. CCA and turf algae exude specific exometabolites to their surrounding environment, and these differentially influence reef microbial dynamics, and biogeochemical parameters, including labile DOC, oxygen availability, bacterial abundance and metabolism [28, 87]. In turn, these could impact the bacterial communities recruited by the coral. CCA-associated bacteria may also produce secondary compounds which trigger variable degrees of coral larval settlement and metamorphosis [75, 79]. These could attract specific bacteria.

Microbiomes of *Pocillopora* recruits and adult colonies

We observed significant differences in bacterial diversity and composition between *Pocillopora* young stages (recruits and juveniles: mean diameter of the colony 13.9 ± 4.82 mm and less than 1 year old) and adult corals. The highest bacterial species richness was associated with the coral recruits and the lowest richness with the adult, which has been observed earlier in *Acropora tenuis* and *A. millepora* [42]. We also observed that the composition of the recruit bacterial community was more variable than the one of the adult. Additionally, the bacterial communities associated with adult corals were dominated by bacterial strains also found in recruits, but in lower proportions, notably the well-known coral endosymbiont *Endozoicomonas* [33, 59]. We further observed an increase in the proportion of recruits-adult common taxa with increasing coral colony size. These results suggest that the establishment of the adult bacterial community may have involved different selection processes from the one of the coral recruit. The highly diverse and heterogeneous bacterial community of recruits may progressively change in the well-established and conserved adult bacterial community comparable to a winnowing process [52], which has been described for the establishment of the *Symbiodinium* symbiosis [1, 41], and with emerging evidence for microbes [4, 40, 42, 69].

Conclusion

Our results provide a new insight into the large diversity of the CCA microbial communities by considerably extending the number of CCA microbiomes studied to date. We show distinct microbiomes, between and within CCA species characterized with distinct chloroplast 16S rRNA gene sequences. We call these distinct groups chlorotypes and demonstrate their utility to differentiate

groups of CCAs. It illustrates the importance of taking into account the host genetics for a better understanding of the microbiome composition. We also show that only few bacterial taxa were shared between the CCA and the coral growing in contact with it. These results suggest that the CCA-algal substrata did not act as the main source for the coral's microbiome. Nevertheless, we observed that the microbial community of coral recruits varied depending on the substrate they grew on. We conclude that CCAs and/or their associated bacteria influence the composition of the coral recruits' microbiome.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40793-024-00640-y>.

Supplementary Material 1

Supplementary Material 2

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Author contributions

LH, XP, RSS, HR, MMN and PEG designed the experiments and collected the samples; CH and PEG analyzed the data and wrote the first draft of the manuscript; HR, MMN, RSS and XP actively commented and corrected the manuscript. HJR conducted the bioinformatics analyses of the raw sequence data; RSS and MMN identified the Crustose Coralline Algae, JP and CB did the sequencing, and managed the repository. BR did DNA extractions.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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