Species-specific microbial communities associated to Crustose Coralline Alga

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Abstract

Crustose Coralline Algae play an important role in reef systems, particularly in larval settlement of invertebrates. CCA present on their surface a biofilm composed by microbial communities which also have a role in the ecosystem. During the settlement phase, larva explore and interact with surfaces to find a substrate to bind. Chemicals cues are needed for the settlement and then the metamorphosis of the larva. This process is called the larval settlement. Several studies have shown that not only do the algae play a role in the larval settlement, their microbial communities are also a key element in this settlement. Other studies have shown that some CCA species can have positive or negative impact on the larval settlement. However, knowing these informations, differences in microbial communities between CCA species are not well documented, or at least, have not been studied in all marine ecosystems around the world. In this paper, we prove that microbial ecosystems associated with CCA (sampled from Ratenbury Island, British Columbia, Canada) are different from the microbial ecosystems living in the water and on the rocks, surrounding these algae. Also, we show that the microbial ecosystems are different between some CCA species. Those results let us think that the interaction CCA-microbial communities are species-specific, and can provide, with further research, more knowledge about the role of the CCA associated microbial communities.

Keywords : microbiomes, reef, larval settlement, 16S rRNA, invertebrates, marine ecosystem

Introduction

Throughout their life cycle, many living organisms associate with microorganisms to get benefits (Rosenberg & Gophna, 2011). Indeed, plants, algae and animals host a multitude of these microorganisms in order to increase their fitness (Faust & Raes, 2012), or to live in extreme ecosystems (Blazejak et al., 2005). One of the most studied microbial communitie is that of humans, particularly the human gut microbiome. It allows us to protect ourselves from pathogens, makes us metabolize important molecules such as sugar, proteins or xenobiotics (Jandhyala et al., 2015). Microbial communities then play a crucial role for their host. However, not only humans host an important microbiome. In this article, we will not touch on the microbiota of human's gut, but we will focus our work on the microbiota in marine environment, the one from the Crustose Coralline Algae (CCA).

Crustose Coralline Algae are red algae that are part of most coral ecosystems (Fabricius & De'ath, 2001). However, CCA are not only limited to corals reefs, they can be found in many marine ecosystems such as artic water (Adey & Macintyre, 1973) or at depths up to 366m (Littler et al., 1985). Currently, 1600 species have been described, but due to the complexity of the determination, it is probably underestimated (Gabrielson et al., 2018). The role of this algae remains to be studied, but there is solid evidence that highlight their importance in reefs ecosystems like reef's accretion (Amado-Filho et al., 2018). Different pelagic animals such as coral, sponges, bivalves, have a larvae phase in their life cycle and need specific chemical cues to identify a suitable substrate to bind and then grow (Siboni et al., 2020). CCA can produce those chemicals and then help invertebrates to bind and then form large reef structure (Littler & Littler, 2013). However, there are also species inhibiting the larval settlement (Siboni et al., 2020). These last years, due to the increase in ocean temperature (White, Lean et al., 1997), and the impact this has on coral reefs (Hoegh-Guldberg, 1999), the role of CCAs and the microbial communities that can be found on it are more and more studied in order to better understand the reef's ecosystem functioning.

Recent studies show that the dead carbonate skeletons of some CCAs' species can induce the larval settlement of coral invertebrates (Heyward & Negri, 1999), but also some bacterias strains isolated from CCA (Negri et al., 2001). It has been shown that microbial communities differ from CCA and their environment (Quinlan et al., 2019). Other studies focus more on the difference of the microbial communities within different CCA species because each species doesn't have the same effect on the larval settlement (Siboni et al., 2020). One of these studies reveal that two species of CCA originating from the Pacific Ocean near Hawaii, *Porolithon onkodes* and *Hydrolithon reinboldii* do not have similar microbial communities (Quinlan et al., 2019). *P. onkodes* facilitate more microbial communities associated with coral disease, whereas *H. reinboldii* facilitate the growth of microbial communities associated with antimicrobial activity. We are then aware that different CCA species have various impact on the larval settlement, that the microbiome plays a role in the creation of coral reef systems, and that microbial communities differ from the one in the surrounding environment of CCA, but also between CCA species. However, this knowledge is not yet deepened and no geographical data or study are known in temperate water.

Contrary to other studies mainly conducted on the Australian coasts (Heyward & Negri, 1999; Negri et al., 2001) or in the warm waters near Hawaii (Quinlan et al., 2019), in this paper, we are interested about the microbial ecosystem of CCAs inhabiting Canada's cold-water seabed at the east side of Ratenbury Island, in British Columbia. We will ask ourselves two questions : Does the microbial communities associated with CCA differ, in composition from the microbial ecosystems living in the surrounding environment of CCA, particularly in water and on the rocks? Is the microbial communities associated with CCA different on various CCA species?

In order to answer those questions, microbial communities were gathered on different substrate and CCA and samples have been sequenced. Sequencing data has been processed and OTUs were assigned to each sample to have an idea of the composition of microbial communities for each of them. This paper provide interesting elements about the microbial communities of three different substrates, but also about different CCA species.

Materials & methods

Data collection

The data collection was performed in August 2019. Boulders were lifted from the seafloor (4-5m deep) on the east side of Ratenbury Island, BC (51°42'03.6"N 128°04'27.4"W) using a fish net and transported back in the lab within 3 hours. Microbiome samples were taken either directly on the boat or in the lab following the following protocol: We rinsed each host alga with sterile seawater in order to remove non-host associated environmental microbes and then sampled microbial communities using a Puritan® sterile swab for 10 seconds. Swabbing was done on an approximate 1*2 cm or otherwise noted. The swab was stored in an individual cryovial (VWR) and placed on ice for transport back to the lab. We collected additional microbial samples from rocky substrate by swabbing bare rock surfaces. We sampled microbial communities from seawater in sterile 500ml plastic containers that were filtered using 0.22µm membrane filter. Filters from each seawater sample, as well the rocks sample and CCA samples were stored at -80˚C.

Molecular Methods

DNA was extracted from swabs and filters using the MoBio PowerSoil®-htp 96 well DNA extraction kit (Carlsbad, CA) following the manufacturer's recommended protocol. Extracted DNA was sent to Integrated Microbiome Resource (IMR), Centre for Comparative Genomics and Evolutionary Bioinformatics (CGEB) at Dalhousie University for PCR amplification and library construction. Primers targeted the V4-V5 region of the 16S rRNA gene for bacteria and archaea, 515f: 5'–GTGYCAGCMGCCGCGGTAA–3' and 926r: 5'–GGACTACNVGGGTWTCTAAT–3' (Comeau, Li, Tremblay, Carmack, & Lovejoy, 2011). Amplicon library preparation and sequencing with Illumina MiSeq using paired-end (2 × 300 bp) v3 chemistry was performed at the Integrated Microbiome Resource at Dalhousie University, in Halifax, Nova Scotia, Canada according to published protocols (Comeau, Douglas, & Langille, 2017).

Bioinformatic processing

The resultant sequences data were processed through the DADA2 pipeline (v1.16) (Callahan et al., 2016) in R 4.0.2 in order to infer amplicon sequence variants (ASVs). Our reads were filtered and trimmed at the position 200 for quality. Forward and reverses reads were then merged in order to construct the full denoised sequence, ASV table was constructed, chimera removed and taxonomy assigned using the Silva reference database. ASVs assigned to "mitochondria" or "chloroplast" were removed and a *phyloseq* object was created to carry further analysis (McMurdie, Holmes, 2013).

Statistical Analysis

In order to analyse the beta diversity, pairwise dissimilarity were calculated using beta-diversity metric (Bray-Curtis distance) taking care of transform data to proportion before , with the *phyloseq* package. Ordination method called Non-Metric Multidimensional scaling (NMDS) (Legendre & Legendre, 2012) based on the Bray- Curtis distance was performed ant then plot with the *ggplot2* package.

A first Permanovas (Anderson, 2017) was performed testing the effect of substrate on the microbial communities, then a second one was performed testing the effect of CCA species on the microbial communities, both using the *adonis* function in the *vegan* package, with 9999 permutations.

Results

Microbial communities differs between substrates

Analyses of the data showed us the five most abundant classes (*Alphaproteobacteria*, *Bacteroidia*, *Cyanobacteriia*, *Gammaproteobacteria* and *Verrucomicrobiae*) for each substrate (Fig. 1a). All three substrate are dominated *Bacteroidia*, *Gammaproteobacteria* and *Alphaproteobacteria*. However, CCA and rocks present more *Cyanobacteria* (~5%) than the water (~1%). Furthermore, CCA host more *Verrucomicrobiae* than the two other substrate.

The 12 most abundant orders are shown in Figure 1b. The six dominant orders of CCA are *Chitinophagales*, *Flavobacteriales*, *Arenicellales*, *Caulobacterlaes*, *Franulosicoccales*, and *Rhodobacterales*. The most dominant order of bacteria occurring on "rocks" and "water" substrates is *Flavobacteriales* (Fig. 1b).

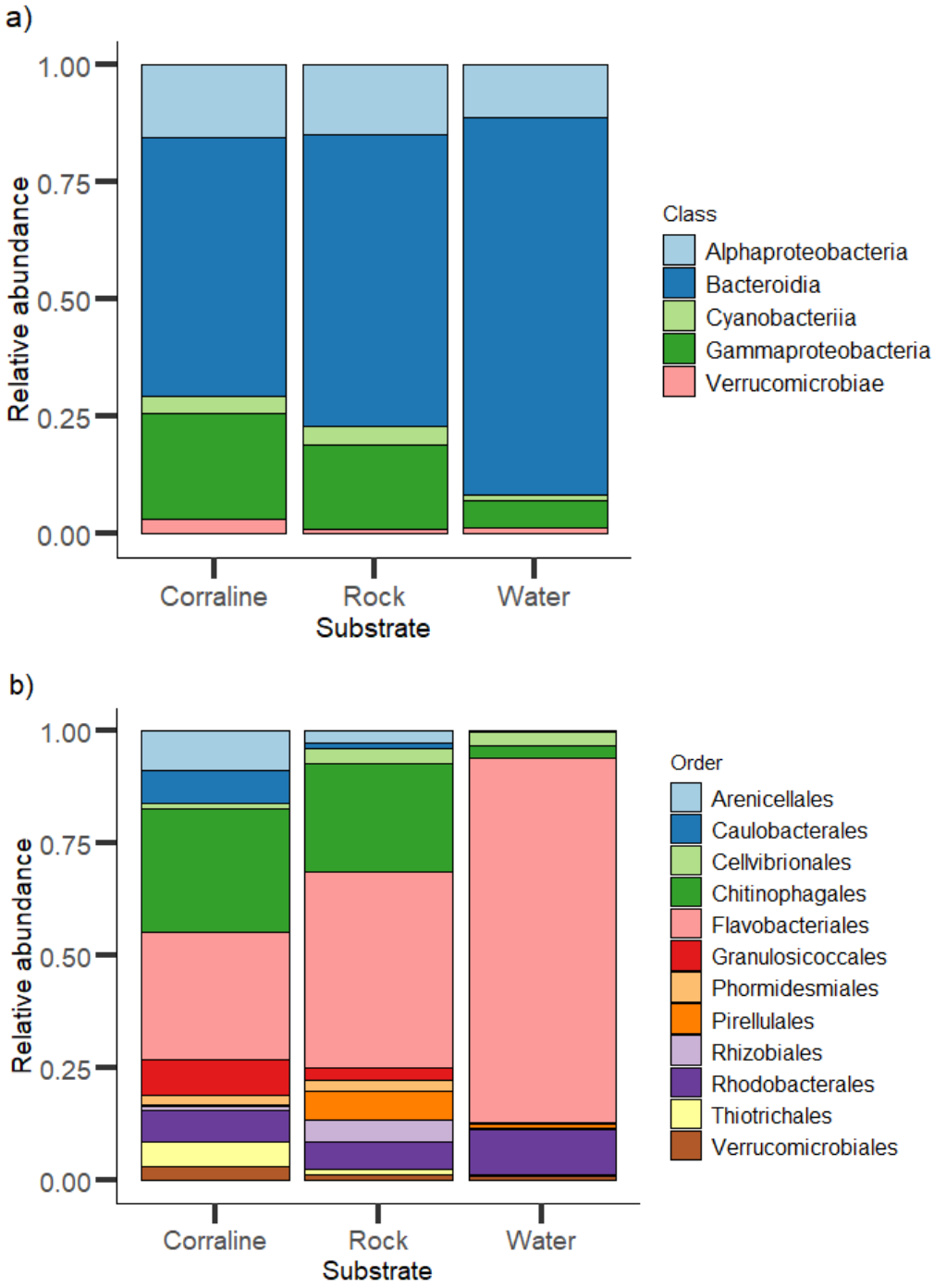

Figure 1 : Variation in microbiota composition across different substrates. Relative abundance were calculated using the proportion of each ASV for each class/order. (a) Class-level microbiota composition by type of substrate (b) Order-level microbiota composition by type of substrate.

The first analysis on Bray-Curtis dissimilarities showing differences of microbiota between substrates (Fig. 2) revealed clear clustering of samples by substrate. The substrate “water” forms a well-defined cluster, while the “rock”, although different from each samples, are well distant from the "water" cluster and the one regrouping CCA. Within this cluster, some CCA samples are away from the center. In addition, the substrate has an significant effect on the microbiota composition (PERMANOVA: substrate, *pseudo*-F = 2.5, P = 1e-04, R2=0.21).

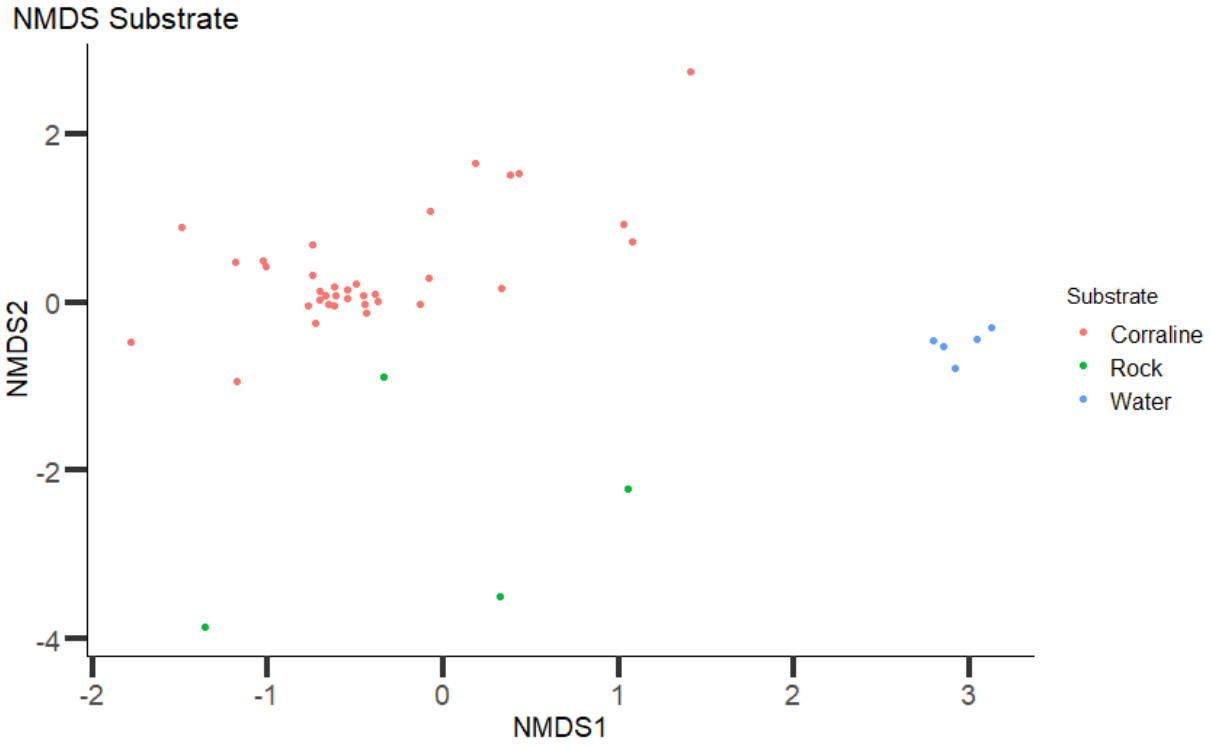

Figure 2 : Clustering of microbial communities in four different substrates, CCA, red crust, rocks and water. Ordination plot with the non-metric multidimensional scaling method (NMDS), based on Bray-Curtis dissimilarity, samples coloured by substrate.

Microbial communities are different between CCA

The second analysis on Bray-Curtis dissimilarities showing the difference of microbiota within the CCA species (Fig. 3) showed clustering for some species. Two *Lithophyllum* species form un cluster between them, *Bossiella* species are well grouped, as well for the *Crusticorallina* species. In addition to this, PERMANOVA test revealed that the species of CCA has an significant effect on the microbiota composition (PERMANOVA: specie, *pseudo-F* = 2.3, *P* = 1e-04, R^2=0.37).

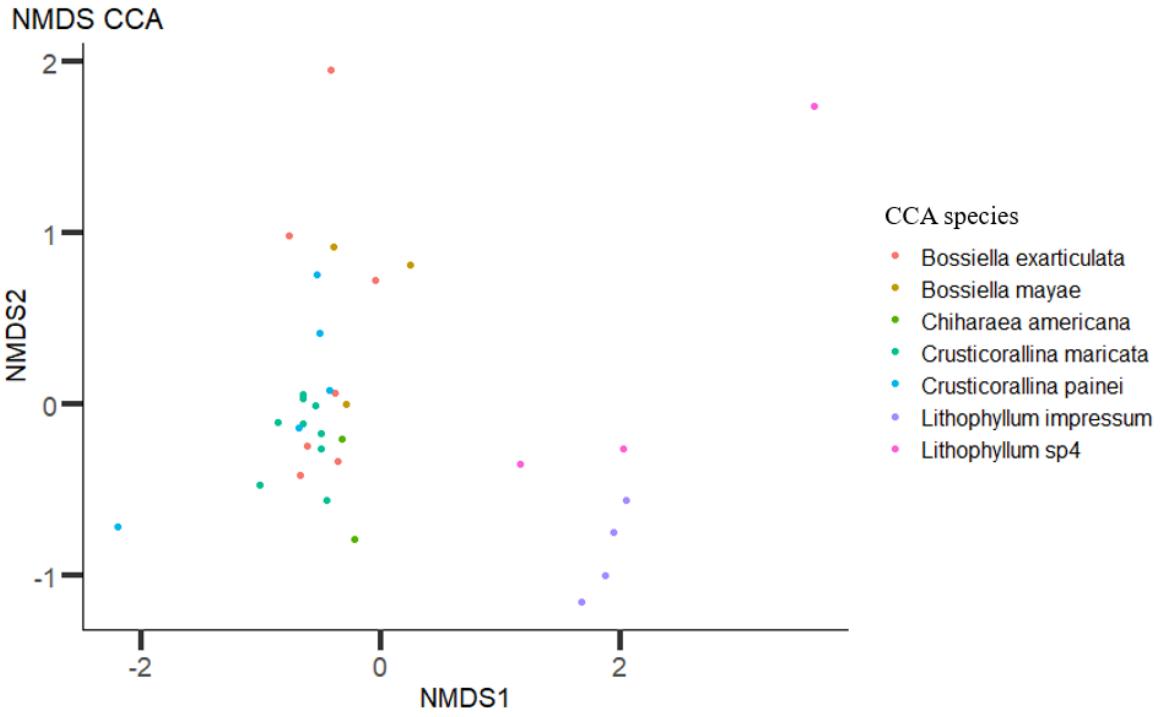

Figure 3 : Clustering of microbial communities in different species of CCA. Ordination plot with the non-metric multidimensional scaling method (NMDS), based on Bray-Curtis dissimilarity, samples coloured by CCA's species.

Discussion

Microbiota taxonomy by substrate

Highlighting the different classes and orders of the microbial communities present in the different substrates allowed us to verify that our samples were not contaminated during collection or handling. The dominant class are *Bacteroidia*, and *Gammaproteobacteria*, which is quite normal given that these are bacterial classes that are very present in the ocean environment (Campbell et al., 2011). The most frequent orders in our CCA samples (F*lavobacteriales*, *Chitinophagales* (S*phingobacteriales)*, *Rhodobacterales*, *Rhizobiales*) are typical of CCA (Quinlan et al., 2019). *Flavobacteriales* is the most abundant order in water. *Flavobacteriales* are well known to have an important role with phytoplankton in oceans, and particularly there are very present in phytoplankton bloom (Pinhassi et al., 2004). *Flavobacteriales* have also an important role in processing of organic matter during phytoplankton blooms (Pinhassi et al., 2004). Those microorganism are also involved in some disease in fishes (Loch & Faisal, 2015). These indications let us know that it's totally normal to find high amount of *Flavobacteriales* in water, because it has different role and impact on the macro and micro ecosystem of oceans. Usual taxa are then find in rock, water and CCA, but CCA and rocks present more various order than water (*Rhizobiales*, *Thiotrichales*, *Granulosicoccales*, *Phormidesmiales)* . Is it possible that some microbial communities have specific needs and require nutriments or specific substrate that can't be find in water. For example, *Phormidesmiales* is known to have an endolithic activity which means that this microorganism need the pore of rocks to survive (Waterworth et al., 2019). Moreover, our study

show that *Thiotrichales* are more abundant on CCA (Fig. 1b), and can be find in very low abundance on rocks. Those bacteria are known to be sulfur-oxidizing bacteria (Hassenrück et al., 2015) and sulfur-oxidizing bacteria have been associated with coral disease (Sato et al., 2017). The fact that CCA induce the larval settlement of coral, and that *Thiotrichales* is a pathogen of corals can explain that we find this order only on CCA. However, there are no coral at the study, but the abundant presence of *Thiotrichales* on our CCA compare to water on the rocks let us think that those bacteria may have a specific interaction between CCA or other animals that rely on CCA. Furthermore, *Granulosicoccales* are also more abundant on CCA. Those bacterias are known to be chemoheterotroph (Lee et al., 2007), which mean that they need to consume organic matter from other organisms in order to live. However, no sign of those bacteria are present in the water, which let us think that CCA certainly produce important nutriments for this bacteria. These biologic facts can explain the differences in microbial communities between substrates show in Figure 2.

Variation of the microbiota communities within CCA

Regarding the variations between CCA species, some of them such as *Lithophyllum* species, have a very distinct microbiome from the other six species. These two species form a cluster together (Fig. 3). For the *Bossiella, Chiharaea,* and *Crusticorallina* species, their microbial communities are quite similar. The fact that *Lithophyllum* species are qui distant from the other species in a phylogenic tree (Aguirre et al., 2010) can explain the difference of microbial communities (Mazel et al., 2018). Furthermore, it has been shown that *Lithophyllum* species have antioxidant and antibiofilm activites, which inhibit the attachment of some microbial communities (Abdulmohsin A. Al-Sofyani, 2017) but no such study was performed on the other species. On explanation about this differences of microbial communities between *Lithophyllum* and *Bossiella, Chiharaea,* and *Crusticorallina* species could be that *Lithophyllum* species host microbial communities who have adapted to the inhibiting compounds produce by the CCA and so those microbial communities are different. This hypothesis concurs with the previous one and with the concept of " phylosymbiosis " (Mazel et al., 2018). Indeed, during the evolution, *Lithophyllum* species may have developed a defence system against unwanted microbial communities and started to produce antioxidant and antibiofilm compounds. In parallel, microbial communities have also adapted to those compound and then a coevolution occurred between the algae and the microbial communities, leading to a differentiation of microbial communities between *Lithophyllum* and the three other CCA species.

Further analysis

Amplicon sequencing only give limit informations on microbiome and can be exposed to different problem when doing PCR or sequencing preparation steps (Poretsky et al., 2014). Silent genes can be amplified during PCR, and thus contaminate the results. Some ASV would therefore correspond to silent genes, which are not representative of the microbial communities' function. Moreover, the concept of species is very controversial in the field of microbiology, some people prefer to refer to the function of a microbial communities than a specie (Burke et al., 2011). Other metagenomic method DNA-based, protein-based can be used instead of amplicon sequencing to have a better understanding of the interactions or functions of microbial communities (Simon & Daniel, 2011).

In this paper, we provide descriptive analysis of microbial communities demonstrating the fact that CCA species have specific microbial communities than their environment, but also that *Lithophyllum* species that we sampled have different microbial communities on their surface than *Bossiella, Chiharaea,* and *Crusticorallina* species. The next step consists in finding the functions of those microbial communities (for each CCA specie) and particularly the secondary metabolites that microbial communities produces. Those functions can help understand the specific role that microbial communities play in reefs ecosystems and their interaction with CCA. It has been shown that larval settlement of a dominant coral species (*Acropora millepora*) in Australia, is predominantly due by chemical cues produce by CCA, but also mediated by interactions between CCA and microbial communities (Gómez-Lemos et al, 2018). In this same study, they showed that microbial communities produce microbial cues but are dependent of the CCA primary metabolism which produce dissolved organic carbon. Further studies need to be done concerning the relationship CCA-microbial communities to examine the role of dissolved organic carbon within microbial communities, for example, using *Pseudoalteromonas* bacterium, which is a bacteria strain that is known to induce larval settlement (Tebben et al., 2015).

Conclusion

In conclusion, through our analysis, we can assume that microbial communities associated with CCA are different from the microbial communities living in the water and on the rocks. Furthermore, the microbial ecosystem is different between some CCA species, particularly for the *Lithophyllum* species.

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