



# Temporal Changes of the Epiphytic Bacteria Community From the Marine Macroalga *Ulva lactuca* (Santa Marta, Colombian-Caribbean)

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## Abstract

Microbial communities live on macroalgal surfaces. The identity and abundance of the bacteria making these epiphytic communities depend on the macroalgal host and the environmental conditions. Macroalgae rely on epiphytic bacteria for basic functions (spore settlement, morphogenesis, growth, and protection against pathogens). However, these marine bacterial-macroalgal associations are still poorly understood for macroalgae inhabiting the Colombian Caribbean. This study aimed at characterizing the epiphytic bacterial community from macroalgae of the species *Ulva lactuca* growing in La Punta de la Loma (Santa Marta, Colombia). We conducted a 16S rRNA gene sequencing-based study of these microbial communities sampled twice a year between 2014 and 2016. Within these communities, the Proteobacteria, Bacteroidetes, Cyanobacteria, Deinococcus-Thermus and Actinobacteria were the most abundant phyla. At low taxonomic levels, we found high variability among epiphytic bacteria from *U. lactuca* and bacterial communities associated with macroalgae from Germany and Australia. We observed differences in the bacterial community composition across years driven by abundance shifts of Rhodobacteraceae, Hyphomonadaceae, and Flavobacteriaceae, probably caused by an increase of seawater temperature. Our results support the need for functional studies of the microbiota associated with *U. lactuca*, a common macroalga in the Colombian Caribbean Sea.

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## Introduction

Microbial communities establish stable associations with eukaryotic hosts and constitute "metaorganisms" called holobionts [1]. Marine macroalgae and epiphytic bacteria exemplify this type of association. In this relationship, macroalgal surfaces have hydrolysable carbohydrates that nourish, epiphytic bacteria [2]. The associated bacteria, in turn, produce beneficial bioactive compounds that influence macroalgal morphology and survival [1].

Macroalgae are home to diverse communities of bacteria with densities varying from  $10^2$  to  $10^7$  cells/cm<sup>-2</sup> of macroalgal tissue [3]. The composition of bacterial communities is influenced by the physiological and biochemical properties of the host, as well as by environmental conditions [4]. Functionally, bacterial communities associated to macroalgae consist of stable and sporadic symbionts. Epiphytic bacteria community composition is shaped by stochastic events or by responses to environmental pressures. Consequently, changes in bacterial community can promote holobiont adaptation to particular environmental conditions [5, 6].

An approach used to study the diversity of environmental samples is the analysis of rRNA genes. Thus, PCR amplification of conserved 16S rRNA gene regions is carried out producing a pool of fragments from communities of microorganisms in a fast and cost-effective way [7]. Further, next generation sequencing platforms play an important role in these studies and make it possible to obtain information to describe the bacterial composition of natural environments [2]. The relationship between epiphytic bacteria and their living host can be studied via a 16S rRNA gene sequencing approach. Sequencing of the 16S rRNA gene has shown that microbial communities from a number of environments (marine, soils, plants, and animals), are much more diverse than previously assumed [8].

Studies of epiphytic bacteria from macroalgae of the *Ulva* genus have identified genes with specific functions in response to conditions in the living host (genes related with the synthesis of metabolites, attachment mechanisms, oxidative stress, heavy metals storage, and desiccation processes). These functions play an important role in the interaction between epiphytic bacteria, eukaryotic communities, and macroalgal surfaces [5].

The epiphytic bacteria community composition of *Ulva* sp. has been described in the Baltic sea and Australia [5, 9–13]. In both regions, communities were mainly influenced by site-specific environmental factors. Furthermore, temporal and spatial comparisons revealed that at lower taxonomic levels, the bacterial community associated with *Ulva australis* varies both among individual macroalgae and across different seasons. In some studies, a community of bacteria was consistently identified across space and time, being deemed as the core bacterial community [10–12]. However, in other studies, such core bacterial community was not observed [5, 14].

As for many living surfaces in the marine environment, little is known about the epiphytic bacterial community from macroalgae inhabiting the Colombian Caribbean. In this study, we describe the epiphytic bacteria community composition of the macroalga *Ulva lactuca*, sampled at La Punta de la Loma (Santa Marta-Colombia) between 2014 and 2016. This bacterial community was characterized through next-generation barcoding of the V3–V4 region of 16S rRNA gene.

## Materials and Methods

### Site Description and Sampling

Macroalgae were collected on the rocky littoral site known as La Punta de la Loma (11°07'00.9"N 74°14'01.3"W), an exposed rocky platform with a macroalgal community dominated by the species *U. lactuca*. This area is influenced by a

rainy period (May–November) that boosts macroalgal diversity, and a dry period (December–April), characterized by a decrease of the abundance and diversity of the macroalgae [15, 16].

Macroalgal blades were sampled in February (during the macroalgal decline period) and in July (during the macroalgal growth period) in 2014, 2015, and 2016, under the Framework Permission (Resolution No. 0255 of March 14, 2014). Up to five macroalgae were randomly collected within a radius of 20 m, where rocky pools were located 1 m apart from one another. Sampling was performed at low tide and macroalgae were removed with sterile forceps and transferred into sterile plastic bags to the laboratory. All *U. lactuca* blades obtained from a single sampling area (in a given month and year) were later pooled in the laboratory to obtain sufficient epiphytic microbial DNA.

### Epiphytic Microbial DNA Extraction

In the laboratory, macroalgal blades were washed three times in sterile seawater to remove attached macroorganisms; subsequently, the blades were cut into sections of approximately 2 cm<sup>2</sup>. Twenty grams of the macroalgal sections were placed into 100 ml of artificial seawater (0.45 M NaCl, 10 mM KCl, 7 mM Na<sub>2</sub>SO<sub>4</sub>, and 0.5 mM NaHCO<sub>3</sub>) supplemented with 10 mM EDTA and 1 ml filter-sterilized rapid multienzyme cleaner (1/100,000) (3 M, Sydney-Australia). Samples were incubated for 2 h at 25 °C and 80 rpm; after incubation, the samples were vortexed for 2 min. The macroalgal material was precipitated by centrifugation at 300 X *g* for 15 min [14] and the supernatant was transferred to new tubes. Epiphytic microorganism DNA was extracted with the ZR Soil Microbe DNA™ kit (Zymo Research Corporation, Tustin-USA) according to the manufacturer's instructions.

To ascertain bacterial removal from macroalgal surfaces, four randomly chosen *U. lactuca* blades were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) prior and after the enzyme treatment. Blade segments were soaked in 1 µg/ml-DAPI in Phosphate-Buffered Saline (PBS) for 30 min; subsequently, DAPI was removed from the macroalgal surfaces by washing three times with PBS [17]. Stained macroalgal segments were placed on slides and viewed under an Olympus B-Max-60 epifluorescence microscope (Olympus Corporation, Tokyo-Japan) with a DAPI Filter (λ<sub>ex</sub> of 358 nm, λ<sub>em</sub> of 461 nm). All images were captured with a 100 X objective.

### Epiphytic Bacteria Community Analysis

Epiphytic microbial DNA concentrations were determined using the QuantiFluor® dsDNA System (Promega Corporation) and then visualized by gel electrophoresis. Microbial DNA from each pool of sampled macroalgal blades (per

month and year) was employed for PCR and sequencing procedures in duplicate. The conditions for PCR amplification of the targeted 16S rRNA gene (regions V3–V4), including modified primers, followed Klindworth et al. [18]. PCR products were single-run sequenced in an Illumina MiSeq sequencer (Illumina, Korea), aiming at generating 300 pb long, paired-end reads of the 16S rRNA gene.

Retrieved forward and reverse read files were trimmed for quality (score < 25). Diversity analyses were performed using the program QIIME 1.9.1: Quantitative Insights into Microbial Ecology [19]. The dataset containing high-quality sequences was then submitted to a chimera detection filter using the Usearch61 method. Selected sequences were clustered into Operational Taxonomic Units (OTUs) using the UCLUST module from QIIME and a pairwise identity threshold of 0.97. Representative sequences for each OTU were picked using the "most-abundant" method. OTU sequence alignment was performed with Pynast [19]. The assignment of each OTU to the closest matching taxon was performed against the SILVA v123 database (<https://www.arb-silva.de>). Sequences matching with eukaryote (i.e. chloroplasts and mitochondria) and archaea genes were excluded from downstream analyses as well as OTUs that occurred less than ten times.

## Statistical Analyses

The OTU table, rarefied to the minimum number of sequences, was used to calculate Chao I, Shannon, and InvSimpson ( $\alpha$ -diversity) indexes. A Kruskal–Wallis tests (*t*-test) was applied to test for significant differences in  $\alpha$ -diversity between months and years of sampling.

The epiphytic bacteria community composition was described at phylum, family, and genus levels, and the relative abundance of these taxa was calculated per sample (February and July, 2014, 2015, and 2016) and ranked by their contribution to the total relative abundance.

Nonmetric multidimensional scaling (nMDS), based on a Bray–Curtis similarity matrix of OTUs relative abundances and cluster analyses, were performed to evaluate epiphytic bacterial community structure differences between *U. lactuca* sampled from the same location at different times [20]. The nMDS approach was carried out using the R package *vegan*, and plots were constructed using the library *Phyloseq* in R [21]. Significant epiphytic bacterial community structure differences between months (February and July) and among years (2014, 2015 and 2016) were assessed with the ANOSIM test, also implemented in the R package *vegan*.

Following Aires et al. [8], three subgroupings were defined for the epiphytic bacterial community studied: (i) core bacterial community, consisting of OTUs present through our study (i.e. in the three years); (ii) variable bacterial community, consisting of OTUs present in two of the

sampling years; and (iii) unique bacterial community, OTUs exclusive of one of the years of sampling. Furthermore, these subgroups of bacterial communities were employed in a Venn diagram, generated with the online tool: <http://www.interactivenn.net/>.

## Nucleotide Sequence Accession Numbers

The nucleotide sequences of the 30 most abundant OTUs were deposited in the GenBank database under accession numbers MF102048–MF102078.

## Results

### Diversity of Epiphytic Bacterial Communities

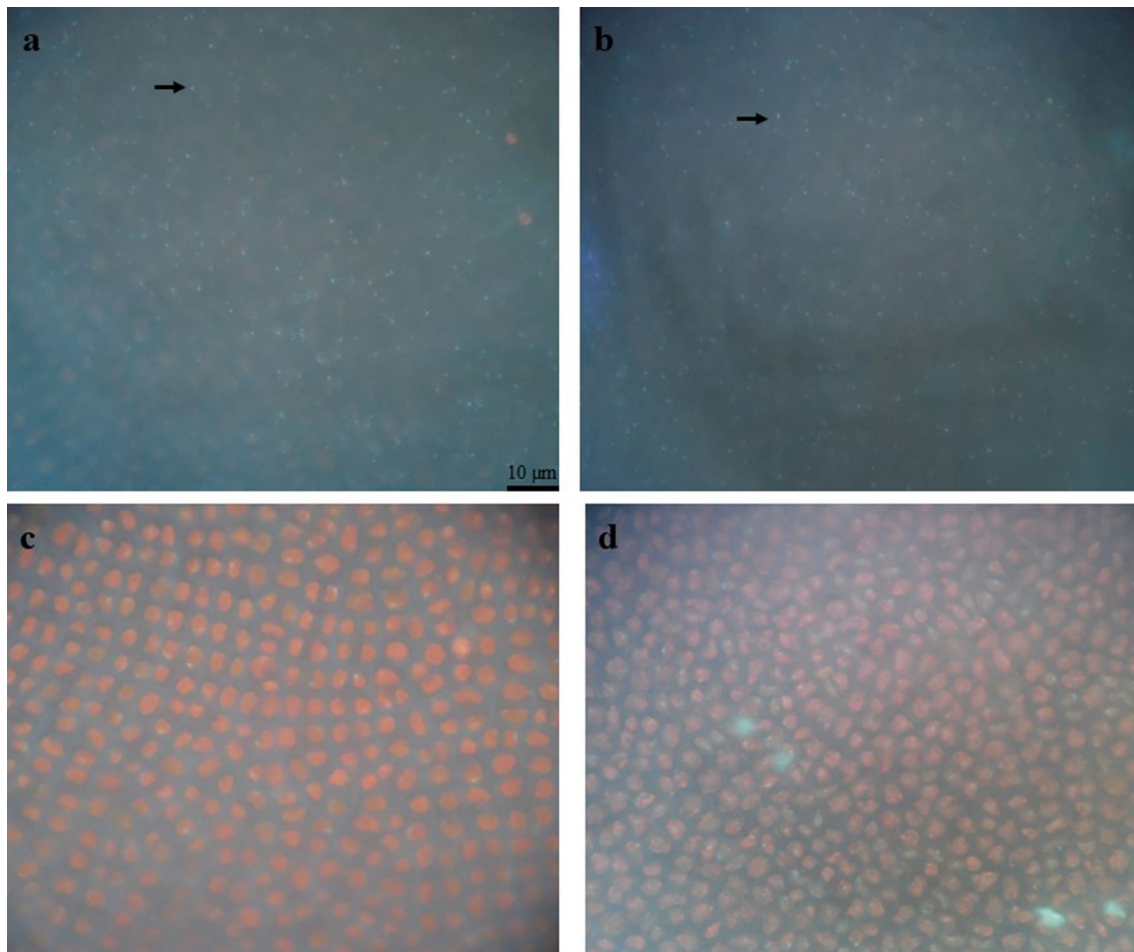
DNA was extracted from epiphytic bacteria on *U. lactuca* blades. DAPI staining of the surface of *U. lactuca* revealed that the sample preparation method led to an almost complete removal of epiphytic bacteria communities, without destroying *U. lactuca* tissues (Fig. 1).

A total of 14,000,000 epiphytic bacteria 16S rRNA gene sequences corresponding to 10,634 bacterial OTUs, were included in downstream analyses after removal of chimeras, chloroplasts, and mitochondria sequences. The highest  $\alpha$ -diversity value was obtained from the 2015 samples (Chao value = 7609) and the lowest from the 2014 samples (Chao value = 5893). However, no significant differences were observed between months (February and July) and years (2014, 2015, and 2016) ( $p > 0.05$ ).

### *U. lactuca* Epiphytic Bacterial Community Composition

A total of thirty-three bacterial phyla were identified in all samples. The epiphytic bacterial communities associated with *U. lactuca* were dominated by the phylum Proteobacteria (44%). Within this phylum, the most abundant classes were *Alphaproteobacteria* (66%) and *Gammaproteobacteria* (26%). The following phyla were also part of the macroalgae-associated bacteria communities: Bacteroidetes (31%), Cyanobacteria (7.4%), Deinococcus-Thermus (4%), Actinobacteria (3.5%), Firmicutes (1.38%), and Chloroflexi (0.85%) (Fig. 2a).

At the family level (Fig. 2b) Flavobacteriaceae was the most abundant family, representing 17% of the epiphytic bacterial community. The families Rhodobacteraceae, Saprospiraceae, and Hyphomonadaceae followed in abundance, with 11.5%, 10%, and 10% respectively. Other important families were the Granulosicoccaceae (5%), Trueperaceae (4%), Flammeovirgaceae (2.5%), Parvularculaceae (2.3%), and Erythrobacteraceae (2.2%).



**Fig. 1** DAPI staining of epiphytic bacteria communities on the blade surface of the macroalga *Ulva lactuca*. Four random blade sections were stained before **a** and **b** and after removal **c** and **d** of epiphytic bacteria communities. Bacterial cells (indicated by an arrow) appear

as light blue dots due to blue fluorescence of DNA-bound DAPI. The red fluorescence is due to the autofluorescence of chloroplasts within macroalgal cells (**c** and **d**). All photographs were taken at 100x (Color figure online)

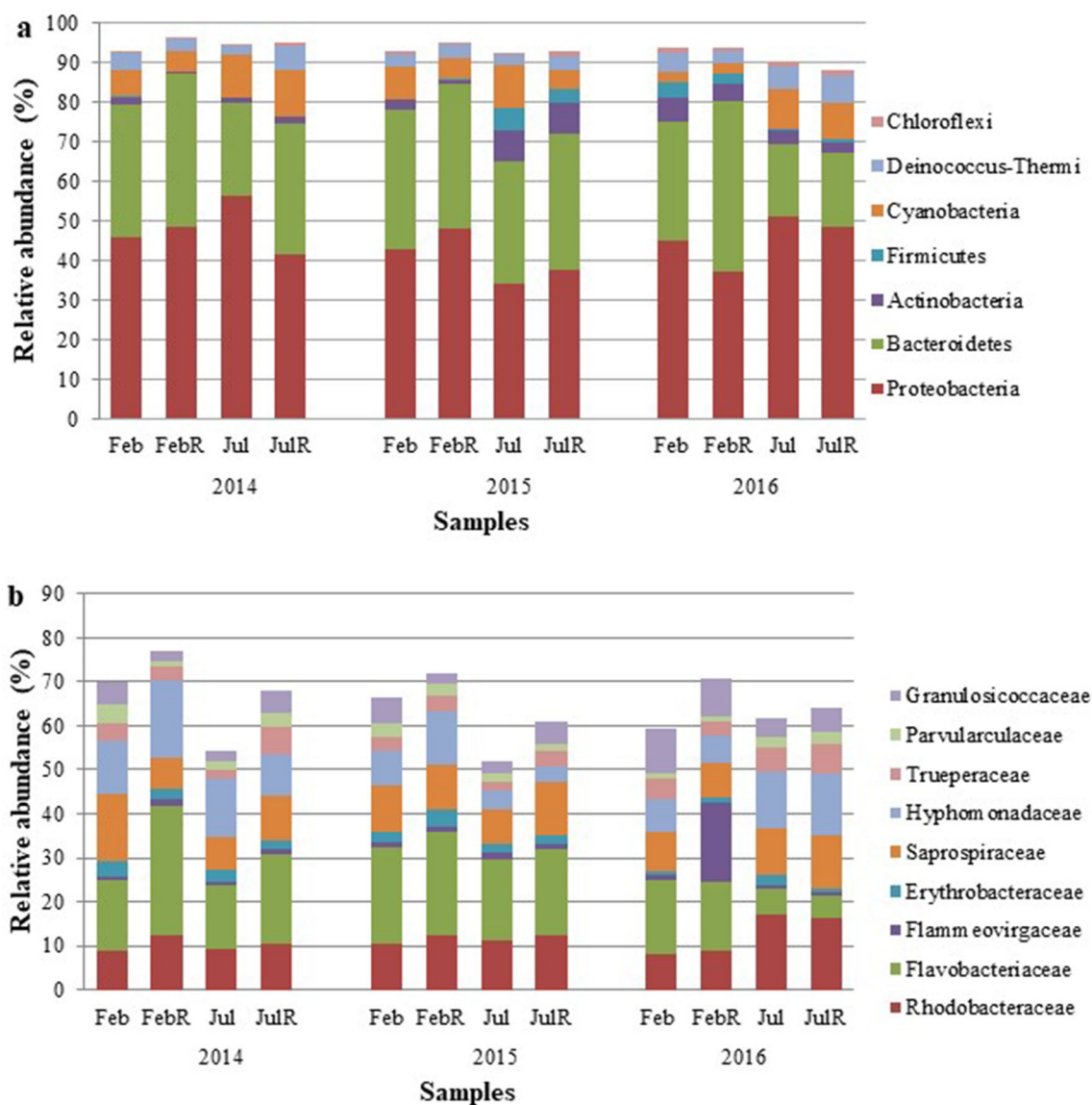
At the genus level, *Algitalea* (5%), *Granolusicoccus* (4.9%), *Aquimarina* (4.5%), *Litorimonas* (4%), *Truepera* (4%), *Hellea* (3.3%), and *Rubidimonas* (2.1%), were the common genera within bacterial communities associated to *U. lactuca* and showed minor changes in relative abundance.

nMDS and ANOSIM analyses revealed that epiphytic bacterial communities on macroalgae, collected in February and July 2014, 2015, and 2016 formed separate clusters (Fig. 3) (ANOSIM analysis:  $R=0.3356$ ,  $P=0.033$ ). Furthermore, the ANOSIM analysis showed similarity among bacterial communities ( $R=0.3356$ ).

The differences observed among sampling years can be explained by changes in the relative abundance of OTUs in the July 2016 sampling with respect to all previous samplings; namely, a marked relative abundance increase of sequences assigned to the families Rhodobacteraceae (16.7%) and Hyphomonadaceae (13.4%), and a small share of sequences assigned to Flavobacteriaceae (5.5%) (Fig. 4).

The comparison of the relative abundance of core, variable, and unique epiphytic bacterial community members revealed that a half of the OTUs (5638, 53%) were shared among all macroalgal samples (Fig. 5). Furthermore, the Venn diagram showed that epiphytic bacterial communities from macroalgae collected in 2014 and 2015 shared the highest number of OTUs (1811, 17%), whereas bacterial communities from macroalgae collected in 2014 and 2016 exhibited the smallest number of shared OTUs (289, 2.7%).

Samplings in 2015 revealed the largest number of unique OTUs (638, 6%). The most common were Flavobacteriales (14.56%), Spingobacteriales (6.86%), Verrucomicrobiales (6.03%), and Cytophagales (2.4%). In 2016, the 472 unique OTUs were represented by Vibrionales (17.7%), Alteromonadales (11.81%), Spingobacteriales (8.5%), Desulfobacteriales (5.08%), and Oceanospirillales (4.30%). The 2014 samplings showed the least number of unique OTUs (85, 0.8%), belonging to the orders Campylobacteriales (10.76%),



**Fig. 2** Relative abundance of **a** phyla and **b** families of epiphytic bacterial communities from *Ulva lactuca*, sampled in February (Feb) and July (Jul) 2014, 2015, and 2016. R: replicate (Color figure online)

Cytophagales (5.58%), Rickettsiales (4.57%), Cellvibrionales (4.10%), and Bdellovibrionales (3.43%). A further 15.75% of the OTUs in this year was unique and could not be identified.

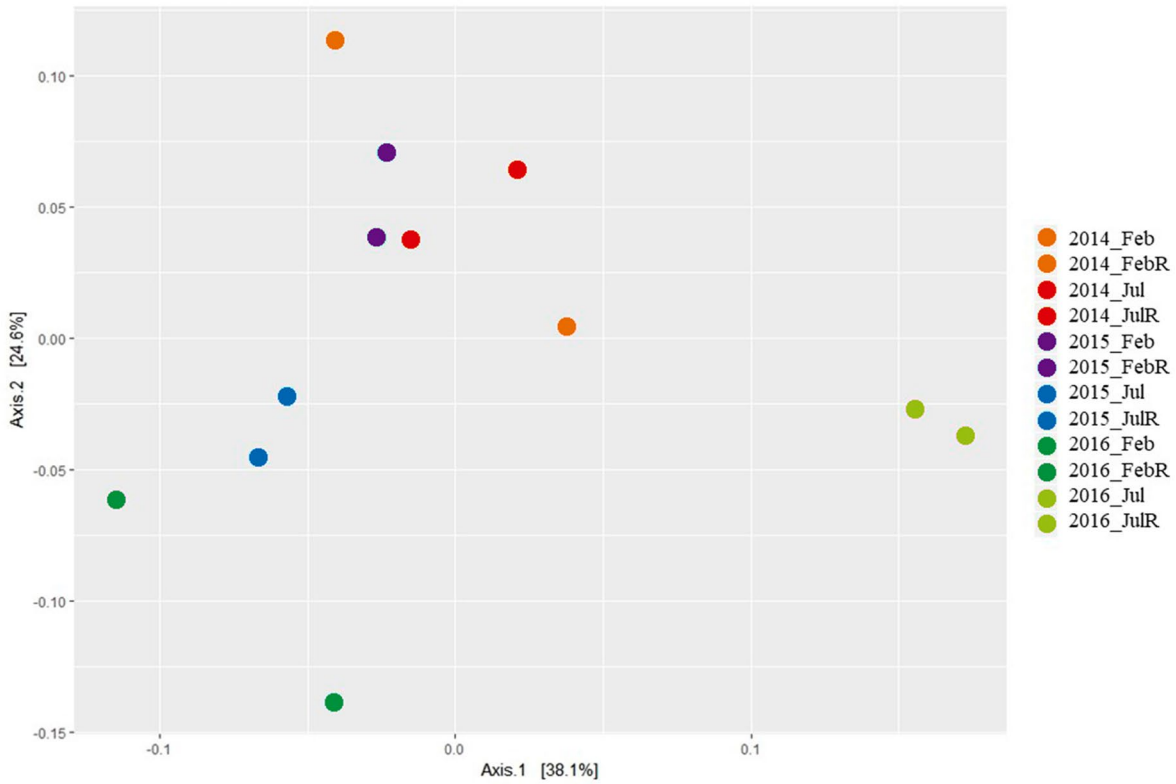
## Discussion

Although several studies have investigated the composition of epiphytic bacteria from macroalgae in different regions [3], little is known about the composition and temporal dynamics of macroalgae-associated bacterial communities from "La Punta de la Loma" (Santa Marta-Colombia). Our study showed a higher abundance of Proteobacteria (classes

Alphaproteobacteria and Gammaproteobacteria), Bacteroidetes, Cyanobacteria, Deinococcus-Thermus, Actinobacteria, Firmicutes and Chloroflexi, resembling bacterial community compositions on *U. intestinalis* from the Baltic sea [10, 12] and *U. australis* from Australia [5, 9, 11, 14].

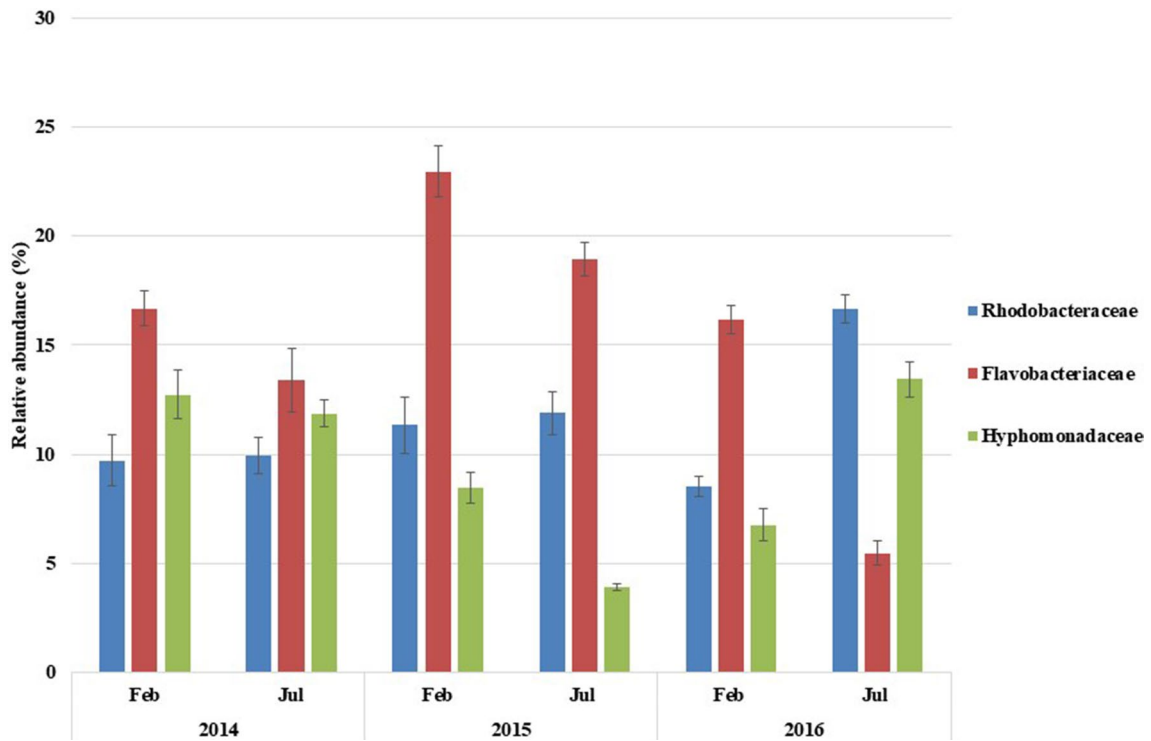
Alphaproteobacteria is a ubiquitous bacterial group in marine environments, hence its dominance in the epiphytic communities studied. Furthermore, most Alphaproteobacteria can assimilate dimethylsulfopropionate, which is produced by macroalgae of the genus *Ulva* sp., as a protection mechanism against high-salinity stress conditions [4, 11].

Bacteroidetes, Cyanobacteria, and Actinobacteria are typical members of marine epiphytic bacterial communities [9]. Deinococcus-Thermus has not been yet reported

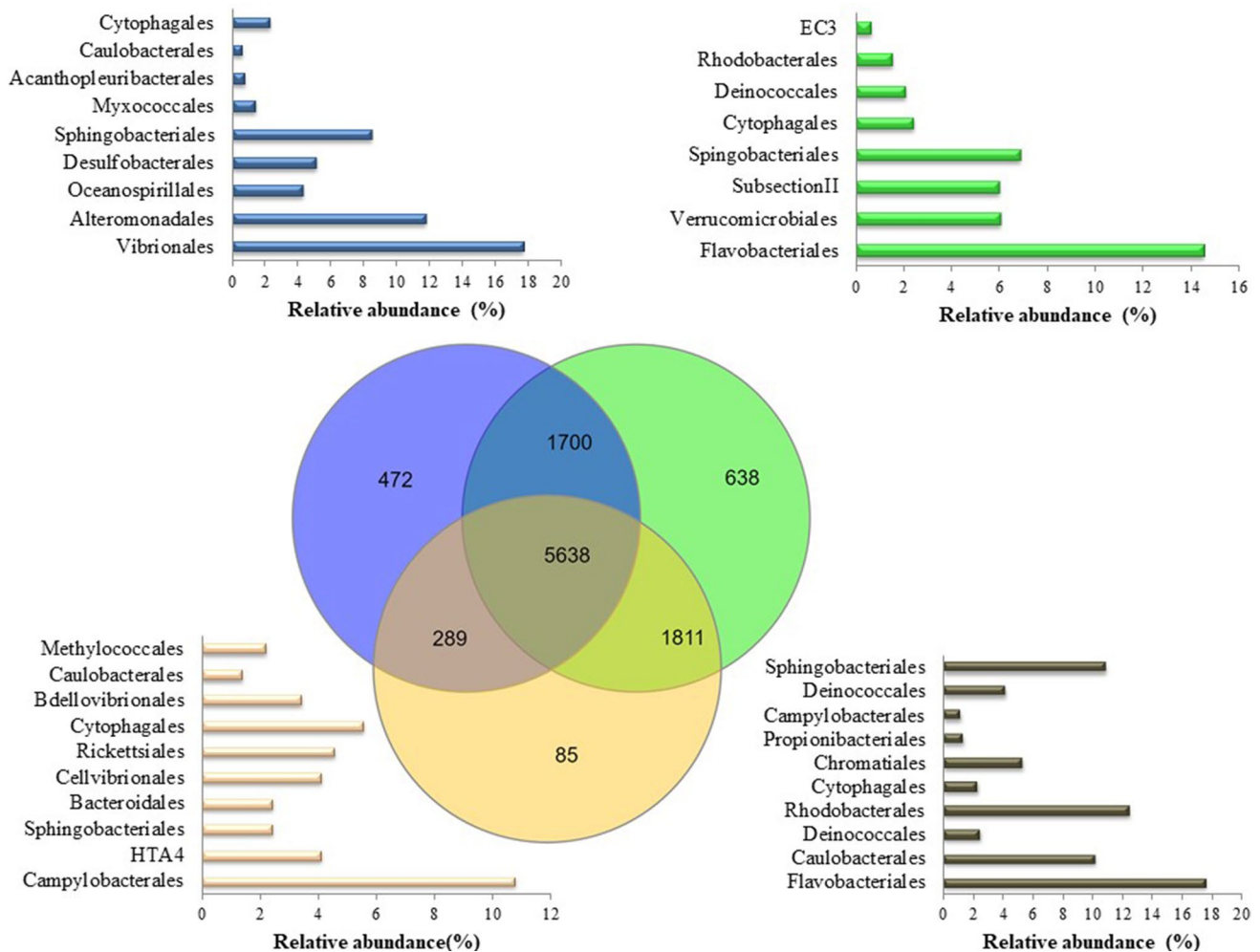


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**Fig. 3** NMDS analyses based on Bray–Curtis similarities among the epiphytic bacterial communities from *Ulva lactuca*, sampled in February (Feb) and July (Jul) 2014, 2015 and 2016, R replicate (Color figure online)



**Fig. 4** Relative abundance for Rhodobacteriaceae, Flavobacteriaceae, and Hyphomonadaceae, in epiphytic bacterial communities from *Ulva lactuca*, sampled in February (Feb) and July (Jul) 2014, 2015, and 2016. R: replicate (Color figure online)



**Fig. 5** Venn diagram representing the number of epiphytic bacterial community members (OTUs) shared among sampling years. Year-specific and core community OTUs (Orders) are shown in bars graphs (beige: 2014, green: 2015, blue: 2016, and gray: core community OTUs) (Color figure online)

associated with *U. lactuca* [9–12, 14], although it can resist extreme radiation and desiccation [22, 23]. The mechanisms that drive *Deinococcus*-*Thermus* association with macroalgae remain to be investigated.

At the family level, bacterial communities from *U. lactuca* were dominated by, Flavobacteriaceae, Saprospiraceae, and Rhodobacteraceae. This result is consistent with studies of *U. australis* [5, 9, 14]. Genomic studies of several marine isolates from Flavobacteriaceae and Rhodobacteraceae reflect adaptation to algal-associated lifestyles [24]. Suites of carbohydrate-active enzyme (CAZyme) genes, organized in clusters, have been described in Flavobacteriaceae. These genes allow Flavobacteriaceae to specialize in polysaccharide available on macroalgal surfaces [25]. Members of Saprospiraceae are not found among free-living organisms. This reflects their propensity to attach to surfaces and their ability to degrade complex nutrients provided by their host [26].

Additionally, Sphingomonadaceae and Planctomycetaceae, two relatively abundant families in bacterial communities associated with *U. australis* and *U. intestinalis*, registered low relative abundances on *U. lactuca*. This is likely because each macroalgal host constitutes a distinct ecological niche with unique biotic and abiotic characteristics that favor the establishment of a particular bacterial group [27].

Furthermore, Hyphomonadaceae, Trueperaceae, Granulosicoccaceae, and Parvularculaceae present in the community of epiphytic bacteria from *U. lactuca*, have not been found on *U. australis* [5, 14]. Members of the Hyphomonadaceae, Trueperaceae, and Granulosicoccaceae are widely distributed in marine environments [28]. Hyphomonadaceae has been reported as part of the coral microbiome of *Porites* sp. corals [29]. Furthermore, some members of this family have antibacterial activity, which can be important in the control of potential pathogens of *U. lactuca* [30]. Trueperaceae was identified in mangrove roots [22, 23], and

Granulosicocaceae was reported in bacterial communities on brown macroalgae [31, 32]. Both families contain heterotrophic bacteria that take advantage of the nutrients available in the surface of *U. lactuca* [33].

When assessed at lower taxonomic levels, epiphytic bacterial communities exhibit high variability [3]. In the present study, the genera *Hellea* and *Algitalea* were identified as members of the bacterial communities on *Ulva* spp. *Hellea* has been found on *U. australis* [11], and *Algitalea* has been found associated with *Ulva pertusa* [34]. Other genera identified in our study, have been described as epiphytes of other marine living hosts: *Litorimonas*, *Granulosicoccus*, and *Tropicimonas* on brown macroalgae [24, 31, 35], *Aquimarina* on red macroalgae [36] and *Rubidimonas* on crustaceans [37].

The composition of epiphytic bacterial communities can vary locally on the same macroalgal host (section of the thallus analyzed) and depends on the chemistry of the macroalgal surface and environmental conditions. Furthermore, methodological factors can also influence the reported composition of epiphytic bacterial communities; for instance, the method to obtain bacterial DNA and the sequencing technique used [3]. Some studies propose that the high variability within bacterial communities among individuals of *U. australis* could be explained by the lottery hypothesis, which proposes that “in a bacterial guild with metabolic abilities to colonize the surface of a macroalga, whichever species from the guild happen to encounter and occupy the surface first are those that will colonize it” [5, 14].

Previous studies argue that the variability, at the genus level, among epiphytic bacterial communities is an emergent feature associated with macroalgae of the genus *Ulva* from different regions: Germany [12] and Australia [5, 14]. In the study of Burke [14] only six out of 528 OTUs characterized from *U. australis* were common to all algal samples. In contrast, our results showed that 226 of the 478 OTUs identified in *U. lactuca* were present across all the samples (Supplementary Fig. S1). Such contrasting results may indicate that epiphytic bacterial communities are perhaps less variable on *U. lactuca* than those on other macroalgae of the *Ulva* genus.

The similarities observed in the composition of the bacterial communities on macroalgae collected in 2014, 2015, and in February 2016 contrasted with the composition of the bacterial community on macroalgae collected in July 2016. This raises the question whether an environmental event could have caused this change. El Niño Southern Oscillation, that took place between 2015–2016, impacting the weather around the world and affecting the coast along the Santa Marta region, was a possible trigger. During the occurrence of this phenomenon, rainfall dropped by 30 to 40% and sea temperatures rose by to 2.5 °C (Institute of Hydrology, Meteorology and Environmental Studies, IDEAM).

Previous studies have shown the deleterious effect of the El Niño on brown macroalgae of the genus *Macrocystis* [38, 39] and this effect has been associated with the increase of seawater temperature and a reduction of nutrient concentration [4, 40].

Further, the increase in water temperature induces a high level of photosynthetic activity and concomitant exudation rates of carbohydrates that can be beneficial for heterotrophic bacteria [41]. This could explain the change in the relative abundances of Rhodobacteraceae and Hyphomonadaceae between February and July 2016. Future studies should address whether changes in temperature and nutrient availability affect community composition on macroalgae of the species *U. lactuca* found in La Punta de la Loma (Santa Marta) Colombia.

## Conclusions

Thanks to a 16S rRNA gene sequencing-based study of the microbial communities living on the macroalga *U. lactuca* from the Colombian Caribbean, we revealed the diversity of these epiphytic bacterial groups. Furthermore, we evidenced that the composition of these bacterial community changed across the years 2014 and 2016.

The composition of the epiphytic bacteria community on *U. lactuca* from the Colombian Caribbean differs from that identified on *U. intestinalis*, in the Baltic Sea and on *U. australis* from Australia. Our results provide insight into the ecology of this bacterial community and motivates further studies on the functional, i.e. transcriptomic and metabolomic, features that shape this marine bacteria-macroalgae association.

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**Author Contributions** Natalia Comba, Liliana López and Dolly Montoya conceived and designed research. Natalia Comba and Nicolás Niño conducted experiments. Natalia Comba, Liliana López and Dolly Montoya analyzed data. Natalia Comba wrote the manuscript. All authors read and approved the manuscript.

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**Data Availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.



## Compliance with Ethical Standards

**Conflicts of interest** The authors declare that they have no conflict of interest.

**Ethics Approval** No animal testing was performed during this study.

**Sampling and Field Studies** All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities and are mentioned in the acknowledgements.

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