Planktonic cyanobacteria belonging to the genus *Synechococcus* are ubiquitously distributed in marine and fresh waters, substantially contributing to total carbon fixation on a global scale. While their ecological relevance is acknowledged, increasing resolution in molecular techniques allows disentangling cyanobacteria’s role at the micro-scale, where complex microbial interactions may drive the overall community assembly. The interplay between phylogenetically different *Synechococcus* clades and their associated bacterial communities can affect their ecological fate and susceptibility to protistan predation. In this study, we experimentally promoted different levels of ecological interaction by mixing two *Synechococcus* ribotypes (MW101C3 and LL) and their associated bacteria, with and without a nanoflagellate grazer (*Poterioochromonas* sp.) in laboratory cultures. The beta-diversity of the *Synechococcus*-associated microbiome in laboratory cultures indicated that the presence of the LL ribotype was the main factor determining community composition changes (41% of total variance), and prevailed over the effect of protistan predation (18% of total variance). Our outcomes also showed that species coexistence and predation may promote microbial diversity, thus highlighting the underrated ecological relevance of such micro-scale factors.

**Key index words:** associated microbiome; coexistence; picocyanobacteria; predation; *Synechococcus*

**Abbreviations:** OTU, operational taxonomic units; PC, phycocyanin-rich cell; PE, phycoerythrin-rich cell

The genus *Synechococcus* is ubiquitously distributed in almost all aquatic environments, owing to a high number of ecotypes or clades with specific adaptation strategies and different lifestyles. The success of these strategies makes this genus particularly effective in shifting environmental conditions. This, combined with its substantial contribution to carbon fixation in oligotrophic aquatic environments (Bell and Kalff 2001, Callieri et al. 2012), highlights the relevance of understanding *Synechococcus* ecological dynamics. Recent studies shed light on distinct niche adaptation mechanisms under varying environmental conditions: but while the evolution and function of marine *Synechococcus* have been more intensively studied in experimental and natural conditions (reviewed in Scanlan 2012), fewer data have been published to date on freshwater *Synechococcus* strains (reviewed in Callieri et al. 2012). Despite this bias toward marine strains, in both environments interactions between cyanobacteria and associated bacterial communities have received comparatively less attention. The intimate association between *Synechococcus* and bacteria observed in the organic matter continuum of oceans (Malfatti and Azam 2009), though, strongly point to the importance of studying these microbial communities in combination, as their interactions at the micro-scale might prove to have broader significance for the success of *Synechococcus* in the environment, expanding our understanding of its adaptations. Supporting this suggestion, experimental evidence has been published linking some cyanobacteria genera to core associated bacterial communities, and showing how laboratory cultures offer good models for environmental studies (Zhu et al. 2016). The relevance of these associations would also seem to find confirmation in the difficulty of cultivating axenic cyanobacterial cultures, owing to the vital role of the associated microbiota, for example, by supplying regenerated nutrients and growth factors (Malfatti and Azam 2009) or removing toxic reactive oxygen species (Morris et al. 2008). Two marine *Prochlorococcus* strains displayed different responses in the presence of heterotrophic bacteria, with antagonistic and enhancing interactions, indicating a potential
influence of the close bacteria-cyanobacteria association on microbial connectivity in the wild (Sher et al. 2011). Unfortunately, most freshwater studies have been performed on potentially toxic cyanobacteria or colonial strains (Li et al. 2012, Shao et al. 2014), leaving the microbial diversity associated to phylogenetically different *Synechococcus* clades largely unexplored.

Against this background, this study begins to explore the role of the heterotrophic bacterial communities living in association with cultures of *Synechococcus* in shaping cell-to-cell interactions. Furthermore, the experiment investigates the subsequent response to predation pressure to identify distinct interactions and pathways underlying microbial niche formation.

Recently, the interaction among different *Synechococcus* ribotypes was proposed as a possible explanation of microcolony formation under predation (Callieri et al. 2016), in relation to the theory of diffusion-sensing when cell-to-cell proximity could facilitate communication (Hense et al. 2007) particularly under predation pressure. A ribotype association with divergent degrees of adaptations could explain the high plasticity of *Synechococcus* in challenging natural conditions. A *Synechococcus*-associated microbiome, differing in composition and diversity, could indicate ribotype-dependent functional interplay between autotrophic cyanobacteria and heterotrophic bacteria.

By experimentally assembling microbial communities with different levels of interactions, we tested two *Synechococcus* ribotypes grown in single culture and co-culture, with and without a nanoflagellate predator, to explore how the ribotype specific associated microbiome responded to species coexistence and protistan predation. Studies on synthetic microbial communities living in association with cultures of *Synechococcus* and protistan predation. To explore how the ribotype specific associations (i.e., cooperation, competition, predation) and that (ii) cyanobacterial identity, in combination with predation, shapes the microbial community.

### MATERIAL AND METHODS

*Experimental set up.* To explore the phylogenetic differences between bacterial communities associated with *Synechococcus* and create different levels of ecological interaction, we set up six semi-continuous treatments (Fig S1 in the Supporting Information). We used two *Synechococcus* strains as preys: strain MW101C3, composed by phycocyanin-rich (PC) cells and belonging to MW101C3 Group I (size: 1.16 ± 0.61 × 0.86 ± 0.45 μm; volume: 0.84 μm$^3$) and strain LL, composed by phycocerythrin-rich (PE) cells and belonging to the Lake Albano Group A (size: 1.52 ± 1.18 × 1.27 ± 0.15 μm; volume: 1.44 μm$^3$; Groebke et al. 2003, Callieri et al. 2013). The *Synechococcus* cultures were not axenic but monoclonal (i.e., derived from one single cell after cell sorting by flow cytometry together with the associated bacteria). The mixotrophic flagellate *Poterioochromonas* sp. strain DS, originally isolated from Lake Constance, kept in axenic culture and fed with dead bacteria as described elsewhere (Blom and Pernthaler 2010, Corno et al. 2013), was used as predator (Hahn and Höfe 1998). *Poterioochromonas* sp. was chosen as it was proven very active in grazing bacteria and picocyanobacteria (Blom et al. 2010, Corno et al. 2013). The *Poterioochromonas* culture was axenic and was added in the specific treatments at a ratio of 1:500 (predator: prey; Corno 2006).

The experiment ran for 4 d in a walk-in chamber at 20°C controlled temperature. All cultures were illuminated with cool white fluorescent tubes at an intensity of 20 μmol photons · m$^{-2}$ · s$^{-1}$ (in 12 h:12 h light:dark cycle). Single cultures of MW101C3, LL, and a co-cultures of the two were incubated with and without predators (six treatments), by using sterilized 130 mL quartz tubes fixed on a slowly rotating device. Each treatment was run in triplicates in BG11 medium (Stanier et al. 1971). The initial concentration of *Synechococcus* was 200–500 × 10$^3$ cells · mL$^{-1}$, similar to the abundances found in oligotrophic lakes (Callieri et al. 2012). The predator was added at the beginning of incubations (T0).

Samples for total cell counting were taken at T0 and after 96 h of incubation (4 d, T4) during logarithmic growth phase. They were immediately fixed (0.2 μm filtered formaldehyde at 2% final concentration) and analyzed by flow cytometry. Samples for Illumina sequencing of 16S rDNA were taken at Tt that corresponds to ~3.5 *Synechococcus* generations, which were sufficient to observe the effects of treatments.

*Flow cytometry.* We quantified *Synechococcus* cells, the predator *Poterioochromonas* and the associated bacteria by the Flow Cytometer Accuri C6 (Becton Dickinson, Oxford, UK), equipped with a 20 mW 488 nm Solid State Blue Laser and a 14.7 mW 640 nm Diode Red Laser. The different pigment composition of cells belonging to MW101C3 and LL generate specific patterns of autoflorescence red signals in the dot plots of channels FL4 (675/25 nm) vs FL3 (>670 nm), thus allowing for their identification, discrimination, and cell counting also when they were mixed together in the co-cultures (Callieri et al. 2016). Cells of *Poterioochromonas* were counted in gates designed on the basis of their specific fluorescence signals and size, in a dot plot of red fluorescence (FL3) vs forward scatter signals. The associated bacterial abundance was obtained by difference between total count and pigmented cell number, after staining with SYBR Green I (1:10,000 final concentration; Thermofisher Scientific, FL1 channel 533/30 nm; Gasol and Moran 2015). Threshold values were set at 1,000 for FL1 and FL3 channels, and at 10,000 for FL4. All data were acquired at a pre-set flow rate of 35 μL · min$^{-1}$, to keep the number of total events below 1,000 · s$^{-1}$.

**DNA extraction and Illumina analyses.** At the end of the experiment, 100 mL were taken from each of the three replicates to obtain sufficient material for the genetic analyses, pool together on 0.2 μm pore-size filter (Nuclepore$^®$) stored at −20°C. DNA was then extracted in duplicate using the UltraClean Microbial DNA extraction kit (MoBio, Carlsbad, CA, USA), the bead beating step by the Precellys 24 (Bertin Technologies, Montigny-le-Bretonneux, France) and minor modifications as described elsewhere (Di Cesare et al. 2015). The variable region V3-V4 of the 16S rDNA gene was chosen for Illumina sequencing using the universal primer pair S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Herlemann et al. 2011). Sequencing was conducted as described by Manzari et al. (2015) on a MiSeq platform. Illumina reads were
RESULTS AND DISCUSSION

Richness. The OTU table was composed by 106 OTUs (Table 1). Richness (Chao 1) was slightly higher in LL compared to MW101C3 (Table 1 and Fig. S2 in the Supporting Information). For both strains, richness was higher under predation and the overall highest number of OTUs was observed in co-cultures, in presence of the predator. Thus, the bacterial community richness increased with the increasing complexity of ecological interactions (in co-cultures under predation, ANOVA with Tukey’s post hoc; Table 2). Our results indicate that the predation of *Poterioochromonas* sp. on different *Synechococcus*-bacteria associations might rely on the number and type of ecological interactions in the newly formed microbiome. Therefore, our findings provide additional experimental evidence supporting the theory that ecological complexity (e.g., competition, allelopathy, predation) maintains high diversity in microbial ecosystems (Mougi and Kon doh 2012).

Bacterial community composition. The two OTUs that were identified as Cyanobacteria constituted between <1% and 8% of all reads, thus considerably less than that measured by flow cytometry (Table 1; Table S1 in the Supporting Information). Due to PCR and extraction bias, the amplicon sequencing cannot provide an accurate estimation of bacterial abundance, such as flow cytometry, and is to be used in a comparative manner (e.g., Aird et al. 2011, Schirmer et al. 2015). Since we focused on the associated bacteria, the two cyanobacterial OTUs were removed for further analysis. The *Synechococcus*-associated bacteria were affiliated with Gammaproteobacteria (*Alkanindiges* and *Pseudomonas*) and Flavobacteria (*Flavobacterium*) in MW101C3 cultures.

Table 1. Cell abundance of *Synechococcus* ribotypes (MW101C3 and LL) (Syn), non-pigmented associated bacteria (bacteria; 10^5 events · mL^{-1} ±SD) and *Poterioochromonas* sp. (*Poterio*; 10^5 events · mL^{-1} ±SD). OTU number (n. OTUs) of associated bacteria and their richness (Chao 1) in all the treatments (single and co-cultures, with and without predation), after 96 h of incubation. The number of cells at the beginning of the experiment (T0) are also reported. Superscript letters indicate significant differences based on one-way ANOVA and post-hoc multiple pairwise comparison among the different treatments (Appendix S1).

Table 2. Differences in Alpha-diversity (Chao 1) depending on the level of complexity of the system, where 1 is single culture, 2 is either co-culture or under predation, and 3 is co-culture and under predation. Table is based on ANOVA with Tukey’s posthoc.
Sphingobacteria (Sediminibacterium), Alphaproteobacteria (Sphingomonas) and Betaproteobacteria (Rhodobacter) in LL cultures (Fig. 1, A and B). Random Forest analysis on a genus level confirmed the importance of Flavobacteria in determining differences between samples that contained the strain MW101C3 (Fig. 1C). On the other hand, Variovorax, Rhodoferax, Sphingopyxis, Sphingomonas and Sediminibacterium characterized treatments with LL and showed the highest relative abundances (>500 reads, Random Forest analysis; Fig. 1C). At OTU level, Random Forest analysis shows that one single Flavobacterium OTU (i.e., OTU 4) was very frequently associated with MW101C3. Two OTUs that characterize the LL treatments were related to Sediminibacterium (Fig. 2), which as Flavobacterium is affiliated with the Bacteroidetes phylum. These results are in agreement with recent literature findings. Zhu et al. (2016) analyzed the bacterial community composition associated to four cyanobacteria genera, reporting on the relatively high abundance of Gammaproteobacteria in pure cultures, similarly to what is found during cyanobacterial blooms in the field. In blooms of Microcystis sp., cyanobacterial colonies were reported to create a perfect habitat for core associated bacteria, which differ from those living in freshwater ecosystems (Parveen et al. 2013). Flavobacterium and Sphingomonas were found in association with cyanobacteria in freshwater systems (Eiler and Bertilsson 2007, Berg et al. 2009, Eckert et al. 2012). Furthermore, Flavobacterium was shown to control allelochemical compounds produced by Poterioochromonas (Blom and Pernthaler 2010). Flavobacterium selective presence in Synechococcus MW101C3 could explain the anti-grazing capability of the microbiome associated to this strain. Most studies were performed on toxic bloom-forming cyanobacteria (e.g., Microcystis spp., Anabaena spp.) and demonstrated how bacteria can inhibit or enhance the growth of cyanobacteria, often resulting in mutualistic association (Berg et al. 2009). In particular, during the bloom sequence, clusters of specific bacteria associated to the bloom were identified (Eiler and Bertilsson 2004, Bagatini et al. 2014).

In the present study, we analyzed for the first time the microbiota associated to Synechococcus
strains. We did not find some of the typical freshwater taxa (e.g., Actinobacteria, freshwater SAR11, Polynucleobacter spp.), because in pure cultures only bacteria that strictly interact with *Synechococcus* are likely to persist. Nevertheless, many of the retrieved genera were also typically found in natural freshwater communities (Newton et al. 2011). The differences found between strains at the distinct interaction levels (treatments) suggested that similar mechanisms may have an underrated role in shaping the aquatic microbial community also under natural condition (e.g., during bloom events; Bagni et al. 2014). While further studies are needed to better understand *Synechococcus*-bacteria interactions, our study offers a first glimpse into which bacteria are strictly associated with *Synechococcus*, and might help in elucidating the role of bacteria in shaping *Synechococcus* niche space.

**Predation.** Bacteria associated with the two *Synechococcus* strains responded differently to predation. Bacteria associated with MW101C3, in the single cultures, were inefficiently grazed. They seemed to be actively removed by predation or outcompeted by interspecific competition in co-cultures, as demonstrated by their significant decrease in cell number after 4 d (Table 1, Table S2 in the Supporting

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**Fig. 2.** Abundances of the six most important OTUs in distinguishing bacterial communities associated to LL and MW101C3 (MW) in all treatments as determined by Random Forest analysis.

**Fig. 3.** Average linkage clustering of the beta-diversity calculated as Sørensen Index. Duplicate extractions (a, b) from experimental treatments. MW: MW101C3; LL: LL; P: *Poterioochromonas* sp.


Table 3. Results of the analysis of variance of the Sørensen distance matrix (ADONIS) comparing the importance of the *Synechococcus* ribotypes (MW101C3 and LL) and predation on Beta-diversity.

|        | F.Model | R²  | P-value |
|--------|---------|-----|---------|
| LL     | 9.3795  | 0.4153 | 0.03125 |
| MW101C3| 2.0324  | 0.08999 | 0.03125 |
| Predation | 4.1727 | 0.18476 | 1       |

Information, Appendix S1 in the Supporting Information. Conversely, bacteria associated to LL were grazed both in single and co-cultures. *Synechococcus* strains responded to predation in a similar way as the associated bacteria. Under grazing pressure single cell cultures and co-cultures responded differently to microcolony formation: MW101C3 formed microcolonies only when in co-culture, while LL formed microcolonies both in single and co-culture. Considering bacteria associated with MW101C3, *Poteriocromonas* caused a strong reduction of bacteria affiliated with *Alkanindiges* (178 times reduced in MW101C3+p compared to MW101C3), only followed by *Hydrogenophaga* which were only two times less (Table S2). Interestingly, whereas *Pseudomonas* related bacteria were not affected by predation in MW101C3, the LL associated *Pseudomonas* were reduced 700 times under predation. *Variovorax* related OTUs were reduced by 100 times, *Hyphomonas* by 80 and *Asticcocaulis* by 47, *Sphingopyxis* by 33 followed by many others (Table S2). Thus, compared to MW101C3, a larger diversity of bacterial genera was affected by predation here. In co-cultures, the main predated genus was the same as in the LL single culture, namely *Pseudomonas* (66 times), followed by many other genera (Table S2).

Factors shaping Beta-diversity. In terms of Beta-diversity (Sørensen-Index), the bacterial community composition of the co-cultures clustered together with the community associated with LL, even when under predation (Fig. 3). Permutational multivariate analysis of variance suggested that predation was not the main cause of the changes occurring within the communities, since it explained only 18% of the variance (Table 3). The presence of LL was the main factor shaping the beta-diversity of the bacterial community (41%), while MW101C3 explained only 9% (Table 3). Thus, a competitive advantage of the bacteria associated with LL over those associated with MW101C3 seemed to be evident.

A different composition of the microbiome associated to *Synechococcus* strains was likely indicating ribotype-dependent functional interplay between the components within the community. Phylogenetic changes of bacterial communities associated to *Synechococcus* ribotypes might help explaining the niche occupation of picocyanobacterial ecotypes at different environmental conditions (e.g., along the vertical or horizontal profile in oceans and lakes;

Tai and Palenik 2009, Callieri et al. 2012). A recent study highlighted the phylum-level specificity of the bacterial communities associated to cyanobacteria, suggesting the use of cultured cyanobacterial strains to study the interactions bacteria-cyanobacteria occurring in natural freshwater blooms (Zhu et al. 2016). These findings, together with our experimental outcomes, increase our confidence in the usefulness of culture models to study *Synechococcus*-associated bacteria interactions.

CONCLUSIONS

In this study, we found distinctive microbiomes associated with *Synechococcus* ribotypes belonging to different phylogenetic clades. Using a simplified experimental system, we observed that the increase of ecological interactions, promoted by species coexistence, affected consistently the diversity profiles of *Synechococcus*-associated microbial communities. Under grazing pressure single cell cultures and co-cultures responded differently to microcolony formation: MW101C3 formed microcolonies only when in co-culture, while LL formed microcolonies both in single and co-culture. In our experimental system, the presence of *Synechococcus* ribotype LL was the main driving factor and prevailed over the effects of protistan predation.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

**Figure S1.** Description of the experimental set up. *Synechococcus* strain MW101C3, composed by phycocyanin-rich (PC) cells and strain LL, composed by phycoerythrin-rich (PE) cells, were used as preys, in single and co-cultures with (+P) or without (−P) predation by the mixotrophic flagellate *Poterioochromonas* sp. strain DS. Further details are provided in Callieri et al. (2016).

**Figure S2.** Venn diagram of OTUs. The left panel compares all OTUs found associated to...
MW and LL in single cultures and co-culture (MW+LL; with and without predation). The right panels compare the effect of predation on single cultures and co-cultures.

**Table S1.** Relative abundance of reads affiliated with Cyanobacteria in the total dataset.

**Table S2.** Changes in the relative abundance of reads affiliated to the most abundant bacterial genera in the data set. Treatments with and without predation are compared. NA: not available.

**Appendix S1.** One-way analysis of variance of *Synechococcus* and bacteria number for the 4 treatments.