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Dynamics of Heterotrophic Bacterial Assemblages within *Synechococcus* Cultures

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ABSTRACT Interactions between photoautotrophic and heterotrophic microorganisms are central to the marine microbial ecosystem. Lab cultures of one of the dominant marine photoautotrophs, *Synechococcus*, have historically been difficult to render axenic, presumably because these bacteria depend upon other organisms to grow under these conditions. These tight associations between *Synechococcus* and heterotrophic bacteria represent a good relevant system to study interspecies interactions. Ten individual *Synechococcus* strains, isolated from eutrophic and oligotrophic waters, were chosen for investigation. Four to six dominant associated heterotrophic bacteria were detected in the liquid cultures of each *Synechococcus* isolate, comprising members of the *Cytophaga-Flavobacteria-Bacteroides* (CFB) group (mainly from *Flavobacteriales* and *Cytophagales*), *Alphaproteobacteria* (mainly from the *Roseobacter* clade), *Gammaproteobacteria* (mainly from the *Alteromonadales* and *Pseudomonadales*), and *Actinobacteria*. The presence of the CFB group, *Gammaproteobacteria*, and *Actinobacteria* showed clear geographic patterns related to the isolation environments of the *Synechococcus* bacteria. An investigation of the population dynamics within a growing culture (XM-24) of one of the isolates, including an evaluation of the proportions of cells that were free-living versus aggregated/attached, revealed interesting patterns for different bacterial groups. In *Synechococcus* sp. strain XM-24 culture, flavobacteria, which was the most abundant group throughout the culture period, tended to be aggregated or attached to the *Synechococcus* cells, whereas the actinobacteria demonstrated a free-living lifestyle, and roseobacters displayed different patterns depending on the culture growth phase. Factors contributing to these succession patterns for the heterotrophs likely include interactions among the culture community members, their relative abilities to utilize different compounds produced by *Synechococcus* cells and changes in the compounds released as culture growth proceeds, and their responses to other changes in the environmental conditions throughout the culture period.

IMPORTANCE Marine microbes exist within an interactive ecological network, and studying their interactions is an important part of understanding their roles in global biogeochemical cycling and the determinants of microbial diversity. In this study, the dynamic relationships between *Synechococcus* spp. and their associated heterotrophic bacteria were investigated. *Synechococcus*-associated heterotrophic bacteria had similar geographic distribution patterns as their "host" and displayed different lifestyles (free-living versus attached/aggregated) according to the *Synechococcus*

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culture growth phases. Combined organic carbon composition and bacterial lifestyle data indicated a potential for succession in carbon utilization patterns by the dominant associated heterotrophic bacteria. Comprehending the interactions between photoautotrophs and heterotrophs and the patterns of organic carbon excretion and utilization is critical to understanding their roles in oceanic biogeochemical cycling.

KEYWORDS *Flavobacteria*, *Roseobacter*, *Synechococcus*, heterotrophic bacteria, metabolic interaction

Microbes play important roles in biogeochemical cycling and productivity in the ocean (1–3). Marine microbes are part of an interacting ecological ecosystem, and comprehending their inter- and intraspecies interactions is critical to understanding their community compositions and distribution patterns in the environment (4–8). Microbes can affect each other through a variety of synergistic interactions (such as cooperation and symbiosis) and antagonistic interactions (such as competition) that influence their function and behavior (5, 6, 9).

Interactions between photoautotrophic and heterotrophic microorganisms are fundamental in the marine food web (1, 10). Heterotrophs utilize photosynthetically fixed organic carbon and other nutrients supplied by photoautotrophs. Free-living heterotrophic bacteria mainly take up labile dissolved organic carbon (DOC) released by photoautotrophs to the surrounding water, while some heterotrophic bacteria attach to photoautotrophs or photoautotroph-derived detrital particles and metabolize particulate organic carbon (POC) as the main sources of organic carbon (10–12). In return, heterotrophic bacteria benefit photoautotrophs by providing essential micronutrients, such as vitamins, amino acids, and bioavailable trace metals (13–17), and reducing the levels of toxic reactive oxygen species, such as hydrogen peroxide (18, 19). Cultures of marine unicellular cyanobacteria, including *Synechococcus* and *Prochlorococcus*, frequently contain associated heterotrophic bacteria, likely due in part to this intimate codependency (20–24). Associations between *Synechococcus* and heterotrophic bacteria were also detected in the environment (25, 26), and geological evidence for their interactions dates to 440 million years ago (mya) (27).

Synechococcus, an ancient and genetically diverse clade, is ubiquitous in the global ocean and is abundant in both estuarine and coastal waters (28, 29). Marine *Synechococcus* members have been classified into three major subclusters (5.1, 5.2, and 5.3), and each of these contains dozens of genotypes (28–30). There are clear geographic distribution patterns for diverse *Synechococcus* clades (29, 30). Those belonging to clades I and IV (subcluster 5.1) are dominant in coastal and higher-latitude regions (31–34). Clade III members (subcluster 5.1) are largely distributed in global oligotrophic waters, similar to clade II (subcluster 5.1) in subtropical/tropical open-ocean waters (29, 32–34). Subcluster 5.2 is typically an estuarine group, with diverse representation in estuaries, such as the Chesapeake Bay (35, 36), whereas subcluster 5.3 members are widely found in the open ocean (e.g., East China, Mediterranean, Sargasso, and South China Seas) (28, 30, 37, 38).

Many different bacteria associate and form close relationships with eukaryotic hosts (e.g., diatoms, corals, and sponges) (10, 13, 39, 40). Compared with eukaryotic entities, how do unicellular *Synechococcus* spp. act as "microbial habitats" and interact with surrounding heterotrophic bacteria? Some heterotrophic bacteria contribute to aggregate formation and particle sinking in diatom cultures (41). During phytoplankton blooms, although the bulk of the bacterial biomass is free-living, the proportion of bacteria attached to algae or particles increases to up to 20% of the total prokaryotes (1). Therefore, investigation of the bacterial community compositions in free-living and attached/aggregated fractions in *Synechococcus* cultures might supply clues for understanding the interactions among these community members and with surrounding environments in natural systems.

The aims of this study were to (i) identify the heterotrophic bacteria that occur in coculture with *Synechococcus* isolates from different environments, (ii) determine

whether the heterotrophic bacteria that were found in coculture with *Synechococcus* isolates have similar geographic distribution patterns as their "host," (iii) characterize the patterns and dynamics in the bacterial community corresponding to different lifestyles (free-living versus attached/aggregated) in a *Synechococcus* culture, and (iv) evaluate the potential for succession in carbon utilization patterns by the dominant associated heterotrophic bacteria in these cultures.

RESULTS AND DISCUSSION

***Synechococcus* isolates.** Ten *Synechococcus* isolates were selected for an investigation of the community structure of their associated heterotrophic bacteria. Five isolates were isolated from coastal/estuary eutrophic waters, and five isolates were isolated from oligotrophic water in the South China Sea (see Table S1 in the supplemental material). Four *Synechococcus* strains (XM-5, XM-11, XM-24, and XM-13) were isolated from the Xiamen coastal/estuary region, and one strain (Cy04) was isolated from the coastal Yellow Sea. Three of the Xiamen *Synechococcus* strains (XM-5, XM-11, and XM-24) belonged to clade CB5 in subcluster 5.2, and the fourth strain (XM-13) was classified into clade IX in subcluster 5.1. Strain Cy04 was grouped into clade VIII in subcluster 5.1 (Table S1). The five oligotrophic *Synechococcus* strains were isolated from the South China Sea. Two strains (YX-A3-2 and ZS02-2) were classified into clade II in subcluster 5.1, and another two strains (YX02-3 and YX04-3) were grouped into clade III in subcluster 5.1. The fifth strain (ZS01-1) belonged to clade V in subcluster 5.1 (Table S1).

According to the classifications of *Synechococcus* in previous studies (28–30, 35), three of the *Synechococcus* strains (XM-5, XM-11, and XM-24) were typical eutrophic estuary genotypes, and four strains (YX-A3-2, ZS02-2, YX02-3, and YX04-3) were oligotrophic water genotypes.

Community composition of *Synechococcus*-associated heterotrophic bacteria. To explore the heterotrophic bacterial community composition associated with *Synechococcus* isolates from different environments, a total of 1,252,549 16S rRNA gene sequences with paired-end 250-bp reads, ranging from 64,389 to 154,972 per sample, with an average length of 225 bp, were obtained from the 10 *Synechococcus* culture samples after passing quality filtering and chimera checks, and these were grouped into 939 operational taxonomic units (OTUs). *Bacteroidetes* (here *Cytophaga-Flavobacteria-Bacteroides* [CFB] group) and *Alphaproteobacteria* and *Gammaproteobacteria* dominated in all samples, and *Actinobacteria* were found in all five eutrophic *Synechococcus* cultures. The top 23 OTU among all samples made up approximately 83% of the total heterotrophic bacteria. These 23 OTU were mostly classified into the CFB group (mainly *Flavobacteriales* and *Cytophagales*), *Alphaproteobacteria* (mainly the *Roseobacter* clade), *Gammaproteobacteria* (mainly *Alteromonadales* and *Pseudomonadales*), and *Actinobacteria* (Fig. 1 and Table S2).

In each *Synechococcus* culture, 1 to 4 dominant OTU (>1% relative abundance) belonging to the CFB group and 1 to 3 dominant alphaproteobacterial OTU were usually found (Fig. 1). At least one dominant gammaproteobacterial OTU was present in all *Synechococcus* cultures, except for strain Cy04. In the strain ZS01-1 culture, one gammaproteobacterial OTU (denovo14255, with 4,289 sequences) accounted for 5.7% of the total heterotrophic bacteria. As mentioned above, *Actinobacteria* were found in all eutrophic *Synechococcus* cultures, with the same actinobacterial OTU found at relatively high abundance in the cultures of the four *Synechococcus* strains isolated from the coastal Xiamen location (Fig. 1). The Cy04 culture also included an OTU (denovo9467, with 4,567 sequences) belonging to the *Actinobacteria*, which accounted for 3.2% of its total heterotrophic bacteria. An evaluation of a coastal Xiamen environmental microbial community assembly showed that the dominant *Synechococcus*-associated heterotrophic bacteria were not abundant in the environmental samples (Table S3), with the exception of denovo10628, which is a member of the *Actinobacteria*, with corresponding 16S sequences widely distributed in the Xiamen coastal area (Table S3). Overall, it appears that the core heterotrophic bacterium assemblages

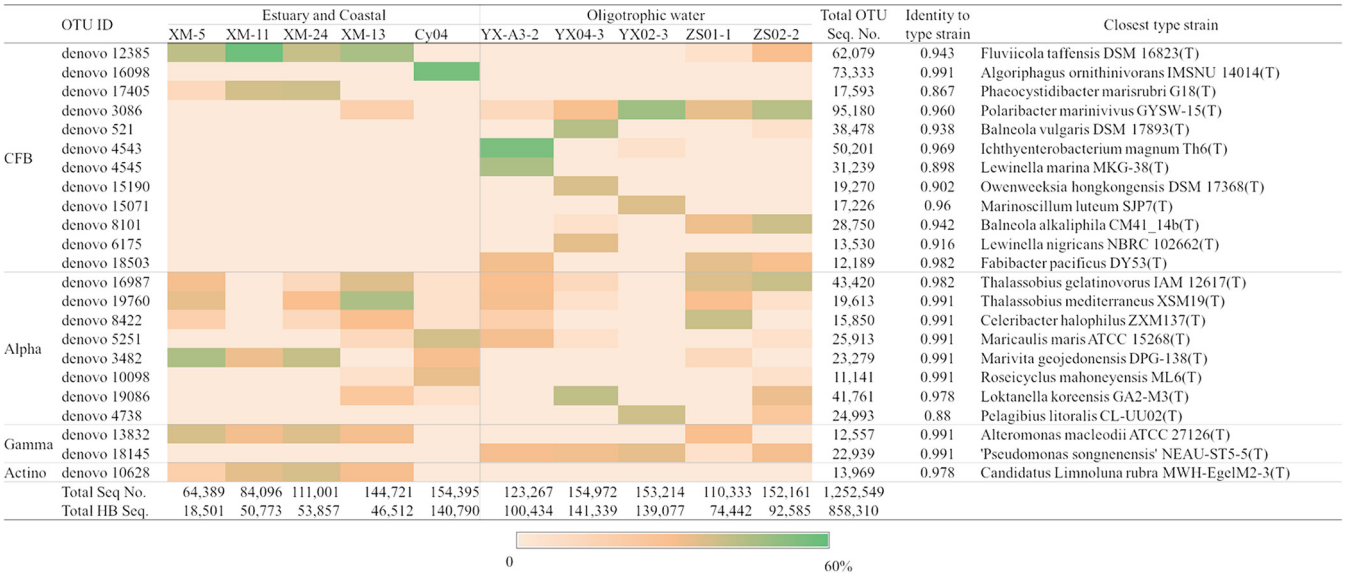

FIG 1 The relative abundances of the dominant heterotrophic bacterial OTUs in each 16S rRNA-sequenced *Synechococcus* culture sample (after removing *Synechococcus* sequences). Total Seq. No., the total number of sequences including *Synechococcus* and heterotrophic bacteria in each sample; Total HB No., total number of heterotrophic bacterial sequences (without *Synechococcus*) in each sample; Total OTU Seq. No., the sum of sequences for each OTU in the 10 samples. Alpha, *Alphaproteobacteria*; Gamma, *Gammaproteobacteria*; Actino, *Actinobacteria*.

comprise at least one species from each of the CFB group, *Alphaproteobacteria*, and *Gammaproteobacteria* in the coculture system, because at least one of each of these was present in every culture (Fig. 1), and *Actinobacteria* regularly associate with eutrophic *Synechococcus* bacteria.

The representative sequences of the dominant OTUs belonging to the CFB group showed much lower identities with type strains, with an average of 94.2% (Fig. 1), in comparison to the representative sequences of dominant OTUs belonging to *Alphaproteobacteria* and *Gammaproteobacteria*, which showed 97.4% and 99.1% average identities to type strains, respectively (Fig. 1). The distant relationships of the dominant CFB sequences to known strains indicate that novel representatives were prevalent in the *Synechococcus* cultures. One possible explanation is that the media used in previous isolation studies could not support the growth of these strains. Another is that these bacteria have been difficult to isolate independently because of their dependency upon *Synechococcus* and/or other heterotrophs.

The CFB OTU denovo12385 was dominant and common in the four Xiamen coastal *Synechococcus* cultures, and it shared 94.3% 16S rRNA gene sequence identity with *Fluviicola taffensis* DSM 16823. *Fluviicola*-related bacteria have frequently been found attached on particles and during phytoplankton blooms (42–44).

The OTU denovo3086 that was dominant and common among the oligotrophic *Synechococcus* strains shared 95.3% 16S rRNA gene identity with *Polaribacter* sp. strain MED152, which is a representative of one of the major genera of *Flavobacteria* within the CFB group (45). Members of the genus *Polaribacter* are more abundant in oceanic regions with higher phytoplankton abundance and show significant correlations with chlorophyll *a* fluorescence (46–48). Genomic analysis showed that strain MED152 possessed a substantial number of genes related to attachment to surfaces or particles and to polymer degradation (45). *Flavobacteria* are thought to be the "first responders" to phytoplankton blooms and can degrade diverse complex high-molecular-weight organic matter by direct attachment and exoenzymatic attack of algal cells and algae-derived detrital particles (11, 49, 50).

OTUs belonging to *Alphaproteobacteria* (mainly in the *Roseobacter* clade) had no clear distribution pattern related to the geographic origins of the *Synechococcus* (Fig. 1), which likely results from their widespread distributions (51–54). Two OTUs (de-

novo16987 and denovo19760) with high identity to members of the genus *Thalassobius* were found in both eutrophic and oligotrophic *Synechococcus* cultures (Fig. 1). The OTU denovo3482 was dominant in three *Synechococcus* cultures isolated from the Xiamen coastal site, and it was also detected in the estuary regions in Xiamen coastal environmental samples collected in the winter (Table S3). Its known relatives, *Marivita* spp., were also found in coastal areas (e.g., Chesapeake Bay and coastal sediments) (55, 56) and phytoplankton cultures, such as those of *Pyrodinium bahamense*, *Coccolithus braarudii*, and *Emiliania huxleyi* (57–59).

The gammaproteobacterial OTU denovo13825, which had high sequence identity to members of the genus *Alteromonas*, was abundant in four Xiamen coastal *Synechococcus* cultures, and it was also frequently found in summer samples from the Xiamen coastal area (Table S3). In contrast, the OTU denovo18145, which was common in the oligotrophic *Synechococcus* cultures, was not identified in the Xiamen coastal environmental samples (Table S3). Members of the genus *Alteromonas* were reported to contribute to the total bacterial biomass at levels similar to those of *Roseobacter* and CFB bacteria in phytoplankton blooms (9, 60, 61).

The actinobacterial OTU denovo10626 was classified into the genus *Aquiluna* and was prevalent in Xiamen coastal microbial communities, especially in the estuary station (Table S3), which indicates they prefer low-salinity eutrophic environments. The presence of *Actinobacteria* accompanying cyanobacterial blooms was frequently detected in diverse aquatic environments, with their relative abundance changing significantly with bloom development (62–64).

In addition to the *Actinobacteria*, the CFB group members and *Gammaproteobacteria* also displayed clear geographic distribution patterns with their host *Synechococcus* isolation environments (Table S1 and Fig. 1). The OTUs denovo10626 (*Actinomycetales*), denovo12385 (*Flavobacteriales*), denovo17405 (*Cytophagales*), and denovo13832 (*Alteromonadales*) were mainly obtained from (at least three) eutrophic *Synechococcus* isolates, whereas OTUs denovo3086 (*Flavobacteriales*), denovo18503 (*Cytophagales*), and denovo18145 (*Pseudomonadales*) were mainly detected from (at least three) oligotrophic isolates.

After examining the community structure of associated heterotrophic bacteria in the *Synechococcus* cultures isolated from different environments, we next sought to investigate the temporal interactions between heterotrophic bacteria and the "host" *Synechococcus* cells. A specific eutrophic *Synechococcus* culture (strain XM-24) was chosen for further analysis. Although we recognize that this study of only one strain limits our ability to know how ubiquitous the observed phenomena are, we believe it is still valuable for probing more deeply into these interactions.

Morphologies of *Synechococcus* and associated heterotrophic bacteria in the *Synechococcus* sp. XM-24 culture. To investigate possible interactions of the *Synechococcus* cells and associated heterotrophic bacteria in the *Synechococcus* sp. XM-24 culture, transmission electron microscopy (TEM) was used to visualize cells from the cocultures. The *Synechococcus* cells were short rods or coccoid-shaped (0.6 ± 0.2 μm by 0.8 ± 0.2 μm) with relatively strong electron density (Fig. S1A). Aggregated cells with different shapes were frequently detected from samples after late-exponential phase (Fig. S1B to D). In addition to the short rods or coccoid-shaped cells, long rod-shaped cells (0.3 ± 0.1 μm by 2 ± 0.5 μm) were also abundant in the cultures. Aggregations of cells in irregular clusters have frequently been observed for many marine bacteria, including cyanobacteria, and these might be caused by changes in the growth conditions and surrounding environments (65). Conjoined *Synechococcus* and heterotrophic bacterial cells were also observed in the marine environment by atomic force microscopy (25, 26).

Dynamic relationship between *Synechococcus* sp. XM-24 and its cocultured heterotrophic bacteria. To characterize the dynamics in the *Synechococcus* culture, the variations in the abundances of *Synechococcus* strain XM-24 and heterotrophic bacteria were determined by flow cytometry. The similar dynamic patterns were observed in the different original inoculum ratios of *Synechococcus* to heterotrophic bacteria ($1.77 \times$

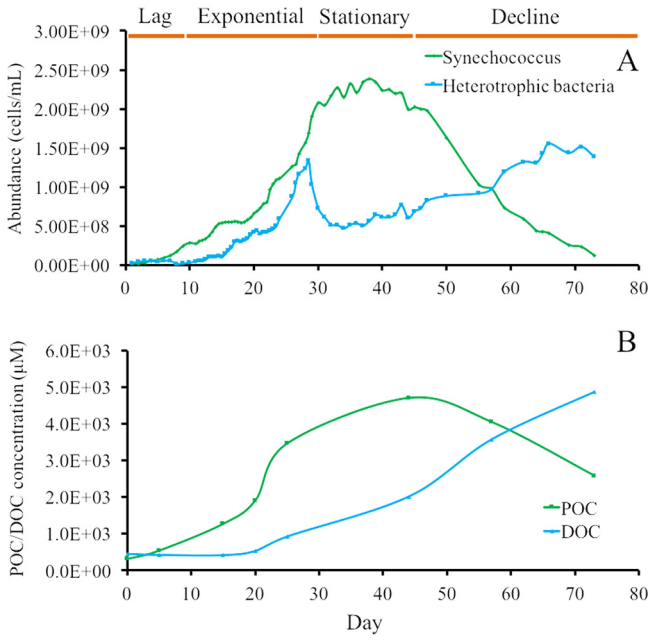

FIG 2 The dynamic relationships between *Synechococcus* and heterotrophic bacteria (A) and POC and DOC (B) in *Synechococcus* sp. XM-24 culture over a 72-day growth period. The abundances of *Synechococcus* and heterotrophic bacteria were measured by flow cytometry. The original abundances of *Synechococcus* and heterotrophic bacteria were 1.76×10^7 and 2.52×10^7 cells · ml^{-1}, respectively. A similar dynamic pattern with different initial inocula of *Synechococcus* sp. XM-24 liquid cultures was observed (Fig. S2 in the supplemental material).

10^7 to 2.52×10^7, 1.54×10^7 to 6.01×10^5 cells · ml^{-1}, and 3.69×10^7 to 1.77×10^7 cells · ml^{-1}, respectively) (Fig. 2A and S2). Therefore, the *Synechococcus* culture described in Fig. 2A was chosen to be representative of the following analyses about dynamic patterns, organic carbon concentrations, and community compositions. The *Synechococcus* strain was inoculated at 1.77×10^7 cells · ml^{-1} and reached a peak density of 2.24×10^9 cells · ml^{-1}. The heterotrophic bacterial abundance ranged from 2.52×10^7 to 1.51×10^9 cells · ml^{-1} over the incubation period. Four phases (lag, exponential, stationary, and decline) were visible from the *Synechococcus* growth curve (Fig. 2A and S2). Interestingly, a sharp decline in heterotrophic bacterial abundance was seen during the *Synechococcus* exponential phase. The heterotrophic bacterial abundance slowly increased when *Synechococcus* began to decline and exceeded *Synechococcus* numbers over the second half of the decline phase. Our preliminary data showed this dynamic relationship was universal among eutrophic *Synechococcus* cultures, whereas a different pattern was found in the oligotrophic *Synechococcus* cultures.

The concentration of POC showed a pattern similar to that of *Synechococcus* abundance, with a rapid increase during the exponential phase and peaking during stationary phase (Fig. 2B). The DOC concentration rose slowly throughout the culture incubation beginning in exponential phase and then surpassed POC in the decline phase.

Although it is not visible in Fig. 2, the abundance of heterotrophic bacteria was slightly higher than that of *Synechococcus* in the early lag phase (2.52×10^7 versus 1.77×10^7 cells · ml^{-1}, respectively). The abundance of *Synechococcus* cells exceeded the heterotrophic bacteria on the fifth day, and this trend continued to the late-decline phase. However, the highest concentration of heterotrophic bacteria was lower than the peak *Synechococcus* number. Based on the correlations of the cell abundances and POC/DOC concentrations, *Synechococcus* was the major source of organic matter in this coculture system, and the labile organic matter consisted of dissolved organic matter (mainly in the exponential and stationary phases) directly released by *Synechococcus* cells and *Synechococcus*-derived detrital particles (mainly in the decline phase).

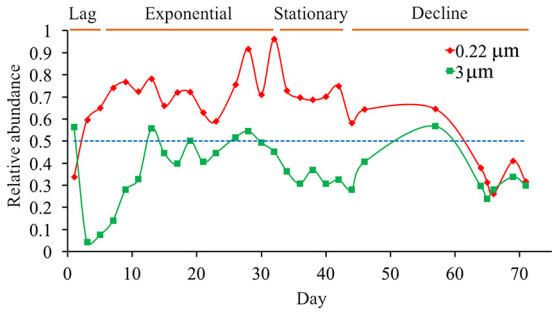

FIG 3 The relative abundances of *Synechococcus* in the total 16S rRNA sequences for each time point from the 0.22- to 3-μm (red) and >3-μm (green) size fractions during the *Synechococcus* sp. XM-24 culture growth. The blue line shows where the relative abundance equals 50%.

During the lag phase, *Synechococcus* and the heterotrophic bacteria tended to aggregate, as most of the cells were in the larger-size fraction (Fig. S3). After the lag phase, the *Synechococcus* bacteria grew exponentially as free-living cells (Fig. S3). In the late-exponential phase, the heterotrophic bacteria tended to attach to *Synechococcus* again, possibly because of biopolymers (such as polysaccharides) secreted by *Synechococcus* (66, 67), which accounted for the sudden decline in heterotrophic bacterial abundance (Fig. 2A). Since the heterotrophic bacterial number was calculated as total bacteria minus *Synechococcus* bacteria, it would be underestimated as result of the attachment or aggregation. After the stationary phase, in the decline period, the *Synechococcus* cells were likely degraded by exoenzymatic attack from certain heterotrophic bacteria (11, 50). Tight associations between *Synechococcus* and the heterotrophic bacteria appeared to be important for the decomposition of organic matter and nutrient cycling in this system (Fig. 2).

Free-living versus attached lifestyles of *Synechococcus* sp. XM-24 over the culture period. To access the different lifestyles (free-living versus attached/aggregated) in the *Synechococcus* culture, sequencing of 16S rRNA gene amplicons from 58 size-fractionated samples collected over the culture growth generated a total of 1,083,985 sequences (530,451 sequences from the 0.22- to 3-μm size fraction and 553,534 sequences from the >3-μm size fraction) with paired-end 450-bp reads that passed the quality control pipeline, with an average length of 392.5 bp.

The average relative abundances of *Synechococcus* in the total 16S rRNA sequences for each size fraction represented 64.3% and 36.9% of total bacteria in the 0.22- to 3-μm and >3-μm size fractions, respectively, over the entire culture growth. Except during the first day, the relative abundance of *Synechococcus* increased rapidly in both size fractions in the lag and early exponential phases and decreased in the decline phase (Fig. 3). After the first day, *Synechococcus* maintained a high relative abundance (>58%) over most of the incubation period, until the decline phase, where it was reduced to <30% of the total bacteria in the 0.22- to 3-μm fraction samples (Fig. 3). In the middle of the exponential phase (from the 13th to the 29th day), *Synechococcus* maintained a high relative abundance (average, 47.7%) in the >3-μm fraction samples. The relative abundance of *Synechococcus* decreased from the late-exponential phase (from the 31st day) and represented only 32.4% of the total bacteria from the 33rd to the 43rd day (during the stationary phase) in the >3-μm fraction samples. A similar trend could be observed in the flow cytometry data, which showed that more than one heterotrophic bacterial cell aggregated or attached to a *Synechococcus* cell at the late-exponential phase, leading to an increased relative abundance of free-living *Synechococcus* and a reduced relative abundance of attached cells (Fig. 3, S3, and S4).

Free-living versus attached lifestyles of the heterotrophic bacteria in the *Synechococcus* sp. XM-24 culture. A total of 193,630 and 351,644 sequences were obtained from the 0.22- to 3-μm size fractions, respectively, after removing *Synechococcus* reads. These sequences were grouped into 380 OTU, which mostly were classified in seven

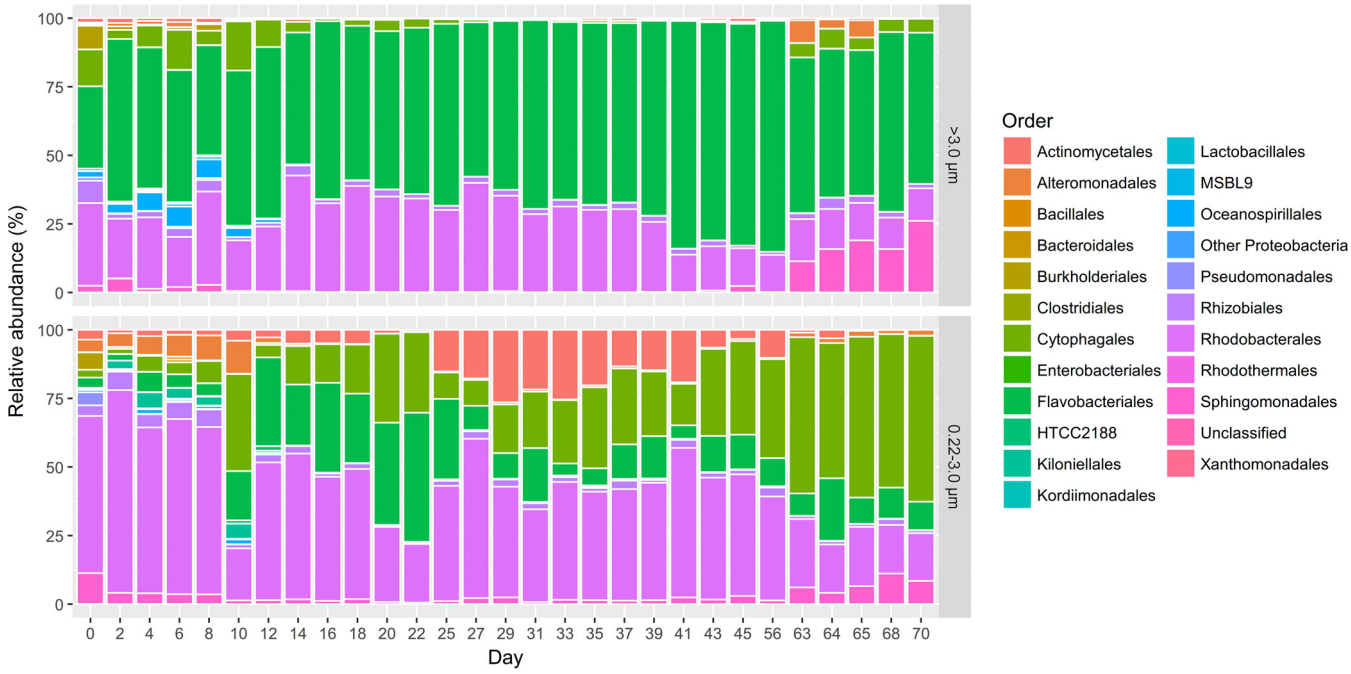

FIG 4 The heterotrophic bacterial community structure from the 0.22- to 3-μm (top) and >3-μm (bottom) size fractions. The relative abundances of different bacterial orders in the total heterotrophic bacterial 16S rRNA sequences (after removing *Synechococcus* sequences) of each time point from the size-fractionated samples collected at different time points are shown.

orders: *Flavobacteriales* (44.2%), *Rhodobacterales* (28.8%), *Cytophagales* (13.1%), *Sphingomonadales* (4.1%), *Actinomycetales* (2.4%), *Rhizobiales* (2.3%), and *Alteromonadales* (1.5%) (Fig. 4). The most numerous OTU in each of these seven orders comprised 66.8 to 97.3% of the total sequences within each respective order, and each of the orders contained 1 to 3 different dominant OTUs (Table S4). Each of the most abundant OTUs contained approximately 3 to 5 dominant genetic types that showed 1 to 3 single-nucleotide polymorphisms, suggesting that each dominant OTU represented a specific strain. As different primer sets were used to investigate microbial assemblages in the 10 cultures versus the XM-24 culture time course, the two sequence data sets could not be put together for analysis. However, the OTUs present in the *Synechococcus* sp. XM-24 culture experiment were only a subset of those identified in the 10 *Synechococcus* cultures in the above-described investigation.

The top 10 OTU made up 89.4% of the total heterotrophic bacterial sequences. Some patterns for lifestyle preference (free-living versus attached) and responses according to the *Synechococcus* growth phases could be discerned (Table S4 and Fig. 5). Two OTUs (OTU1 and OTU4) belonging to the *Flavobacteria* were most abundant in the attached/aggregated fraction (Table S4 and Fig. 5). OTU1 (corresponding to denovo12385 in Fig. 1), which is classified into the order *Flavobacteriales* and genus *Fluviicola*, was the most dominant heterotrophic bacterial OTU. Its relative abundance from the >3-μm fraction was much higher than from the 0.22- to 3-μm fraction over the entire incubation (Fig. 5). Its relative abundance in the 0.22- to 3-μm fraction did increase during the exponential phase, with lower abundance in the other three phases. However, its relative abundance in the >3-μm fraction increased over the entire incubation until the decline phase, when it decreased quickly.

The second most dominant OTU (OTU2, corresponding to denovo3482 in Fig. 1), which belongs to the genus *Marivita* in the *Roseobacter* clade, shifted between free-living and attached over the culture growth period (Table S4 and Fig. 5). In the lag and decline phases, it was more abundant in the free-living fraction, while its distribution was more even during the exponential and stationary phases. This pattern is consistent with the known abilities of related bacteria to metabolize biopolymers generated by

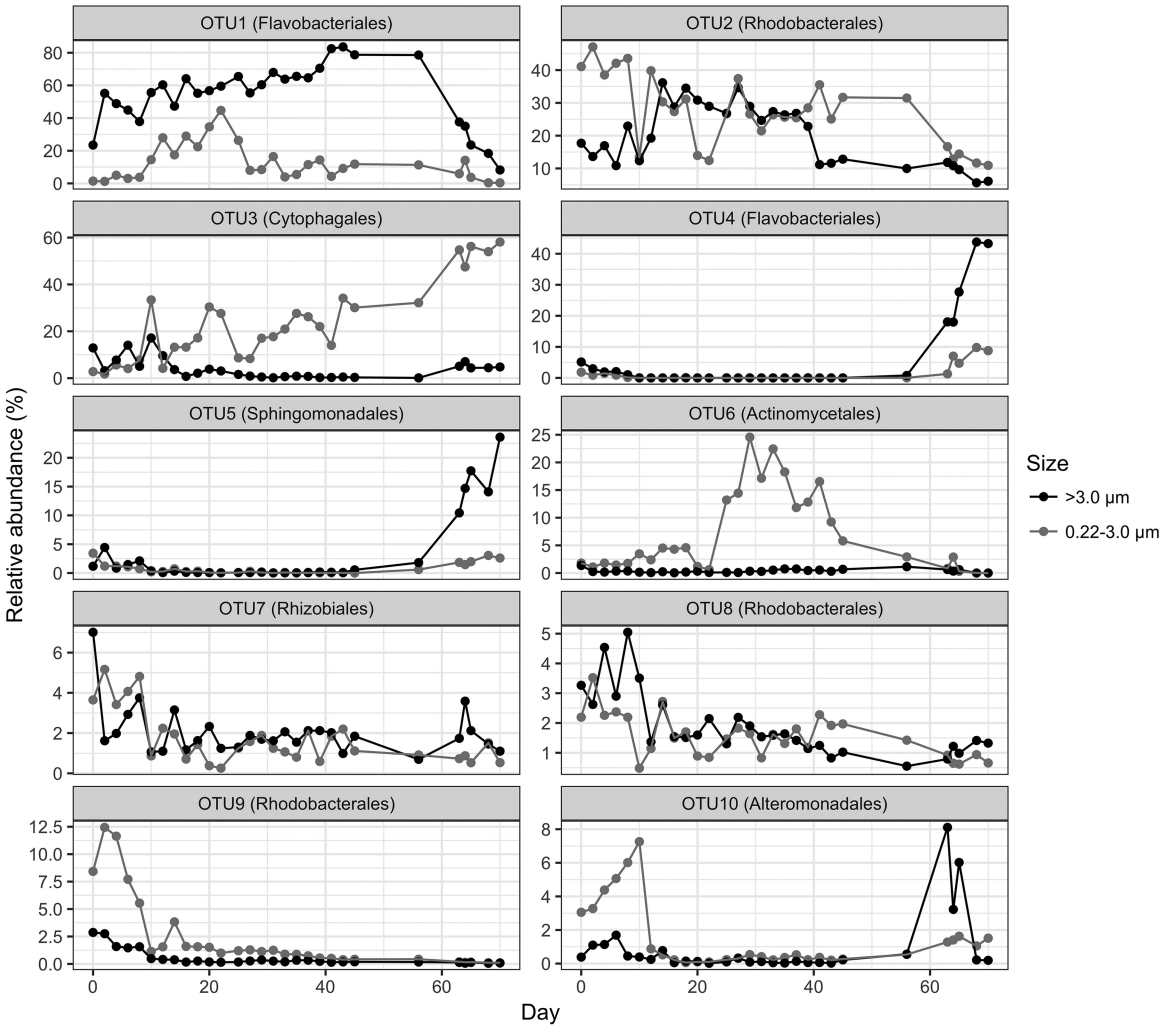

FIG 5 Comparison of relative abundances of the top 10 heterotrophic bacterial OTUs from the 0.22- to 3-μm and >3-μm size fractions over the *Synechococcus* sp. XM-24 culture growth period. OTUs 1 and 4, order *Flavobacteriales*; OTUs 2, 8, and 9, order *Rhodobacterales*; OTU 3, order *Cytophagales*; OTU 5, *Sphingomonadales*; OTU6, order *Actinomycetales*; OTU7, order *Rhizobiales*; OTU10, order *Alteromonadales*.

marine phytoplankton using hydrolytic enzymes (17) and to directly import low-molecular-weight DOC (53, 68).

The third most dominant OTU (OTU3, corresponding to denovo17405 in Fig. 1), which belonged to the genus *Roseivirga* in the order *Cytophagales*, was most abundant in the free-living fraction (Table S4 and Fig. 5). Its relative abundance from the 0.22- to 3-μm fraction surpassed that from >3-μm fraction in the early exponential phase and increased greatly in the decline phase, and it had relatively low relative abundance in the >3-μm fraction in all four growth phases. The lifestyles of the three most dominant OTUs were also proven in another two incubation systems of *Synechococcus* sp. XM-24 cultures (Fig. S4).

Both OTU4 and OTU5, belonging to the orders *Flavobacteriales* and *Sphingomonadales*, respectively, had a clear response in the decline phase (Table S4 and Fig. 5), and the response in the >3-μm fraction was much stronger than in the 0.22- to 3-μm size fraction. This indicates that these bacteria grow quickly by direct attachment to other microbial cells or derived detrital particles. Members of the *Sphingomonadales* are prevalent within phytoplankton blooms and are abundant in particle-attached samples (69–72).

OTU6 (corresponding to denovo10628 in Fig. 1), which was classified into the genus *Aquiluna* in the order *Actinomycetales*, was mainly found in the 0.22- to 3-μm size

fraction and had higher relative abundance in the late-exponential and stationary phases (Table S4 and Fig. 5). This corresponds to when the *Synechococcus* cells began to release more DOC (Fig. 2). Although there was a much higher DOC concentration in the decline phase, most of this represents by-products from *Synechococcus* and the degradation of other bacterial cells. This suggests that this actinobacterium might prefer fresh DOC released by *Synechococcus*. Previous studies showed that *Actinobacteria* could be found at high abundance in both free-living and particle-attached lifestyles, especially in temperate freshwater ecosystems (73, 74), whereas others have suggested that actinobacterial abundance is significantly higher in free-living than in particle-attached states (37, 75). The high diversity within *Actinobacteria* might account for different lifestyles (76). However, there was only one group of *Actinobacteria* found in these *Synechococcus* cultures, and they were most similar to the known "*Candidatus* Aquiluna" sp. strain IMCC13023 that possesses a relatively small genome size (1.36 Mb) (77).

Both OTU7 and OTU8, which belonged to the orders *Rhizobiales* and *Rhodobacterales*, respectively, showed no differences in their relative abundances between the two size fractions, although both were in relatively high abundance in the lag phase. OTU9, belonging to the *Rhodobacterales*, showed higher relative abundance in the early growth stage in the free-living fraction. OTU10, corresponding to denovo13832 in Table S4 and classified in the order *Alteromonadales*, was abundant in the free-living fraction at the early growth stage and then in the attached fraction late in the decline phase (Table S4 and Fig. 5).

Some of the heterotrophic bacteria had obvious preferences for a free-living (e.g., OTU3 and OTU6) or attached/aggregated (e.g., OTU1) lifestyle. Others, such as OTU2, shifted between the two different lifestyles. Similarly, some environmental microbes have been found to occur in different size fractions (78, 79). Therefore, these lifestyles are not only determined by the bacterial genetic features but also by changes in the surrounding environment.

Origin of *Synechococcus*-associated heterotrophic bacteria. In this work, some of the *Synechococcus* strains were isolated using dilution methods on plates, which have a low efficiency of isolation success, and continuous dilution streaking of the *Synechococcus* onto plates failed to produce axenic isolates. It was previously found that axenic cultures of some cyanobacteria, obtained by antibiotic treatment, grew efficiently only if the inoculum was large (14, 18, 19). This would help explain the difficulty in obtaining *Synechococcus* colonies using dilution methods, where the inoculum is much lower. However, previous studies showed that colonies of cyanobacteria formed easily when they were cocultured with heterotrophic bacteria that helped the cyanobacteria reduce oxidative stress (14, 18). In addition, *Synechococcus* bacteria were observed as being conjoined with heterotrophic bacteria in the environment (25, 26). In line with this logic and going through the isolation process used here, at the early stage of dilution and spreading of the water samples on the plates, the *Synechococcus* bacteria interacting with heterotrophic bacteria would be able to grow (Fig. S5). A few heterotrophic bacteria might be able to utilize agar components and form small colonies on the plate (Fig. S5), but most marine heterotrophic bacteria will not grow on the SN medium (95) employed unless they are associated with growing *Synechococcus* cells. After the *Synechococcus* colonies begin growing, organic material is released around the colonies, and the associated heterotrophic bacteria in the plate continue to survive and grow (Fig. S5). The heterotrophic bacteria form close associations with and attach to the *Synechococcus* cells. In order to obtain additional fresh organic matter, the heterotrophic bacteria that were utilizing agar components or surviving through other means might also attach to the *Synechococcus* colonies (Fig. S5). These original heterotrophic bacteria that facilitate *Synechococcus* colony formation and that attach and grow with the colony represent the *Synechococcus*-associated heterotrophic bacteria that were present in the subsequent liquid cultures.

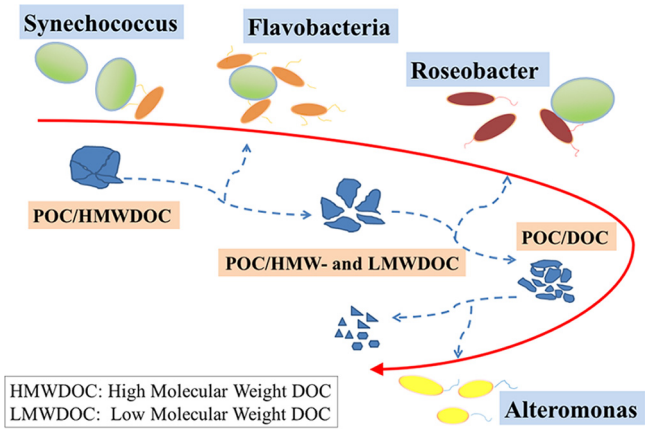

FIG 6 Schematic representation of the patterns of utilization of the organic matter produced by *Synechococcus* by different heterotrophic bacteria. HMWDOC, high-molecular-weight DOC; LMWDOC, low-molecular-weight DOC. *Flavobacteria* prefer an attached lifestyle in the *Synechococcus* culture and can degrade POC and complex HMWDOC. Roseobacters shift between free-living and attached lifestyles, and they not only utilize POC/HMWDOC but also uptake labile LMWDOC, including by-products from the growth of *Synechococcus* and *Flavobacteria*. *Alteromonas* demonstrates broad substrate preferences and may use different complementary growth strategies relative to the other two bacterial groups.

It is possible that the agar itself is a potential carbon source for some marine heterotrophic bacteria, and the agar concentration used for the *Synechococcus* isolations might affect heterotrophic bacterial growth and composition. In addition, the different capacities with respect to motility and chemotaxis of heterotrophic bacteria on the agar plates might impact the formation of the heterotrophic bacterial pool.

In the liquid culture, the bacteria responded to multiple selection pressures, and some became abundant at different growth phases. The tight associations between *Synechococcus* and the heterotrophic bacteria reflect the complementation of physiological or biochemical functions, and the resultant heterotrophic bacterial community structure presumably allows for optimal metabolic performance and enhanced productivity.

Community succession during utilization of organic matter from *Synechococcus* cells. In order to easily understand and visualize the interactions between *Synechococcus* and associated dominant heterotrophic bacteria, a conceptual model was built based on the different lifestyles employed over the culture growth and on carbon availability and utilization changes in the cultures (Fig. 6). It displays a successional utilization relationship related to the organic matter availability. This agrees with previous publications (10, 50). Analysis of the associated heterotrophic bacterial assemblages indicates that at least one species from each of the *Flavobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* consists of the core and abundant components in the *Synechococcus* coculture system.

Flavobacteria, which were the most abundant group and preferred an attached lifestyle in the *Synechococcus* cultures (Fig. 1 and 5), seem to be specialized in the degradation of complex high-molecular-weight organic matter (Fig. 6) (45, 50, 80, 81). Members of the *Roseobacter* lineage, the second-most dominant group in the cultures, shifted between free-living and attached lifestyles. As ecological generalists, roseobacters not only utilize biopolymers with hydrolytic enzymes (17) but also uptake labile low-molecular-weight DOC, including by-products from the growth of *Synechococcus* and *Flavobacteria* (Fig. 6) (50, 53, 68). Marine *Gammaproteobacteria*, specifically members of the *Alteromonadales*, demonstrate broad substrate preferences (82, 83) and have obvious responses during phytoplankton blooms (9, 60, 61). According to their behavior in the *Synechococcus* cultures in this study, with the largest responses in the early growth stage and decline phase, they cycled opposite of the *Flavobacteria* and roseobacters, suggesting they may use different complementary growth strategies relative to the other two dominant bacterial groups.

Cyanobacteria contribute approximately 40% of the global ocean primary production (84–87), and organic matter excretion by these primary producers drives carbon cycling in the upper ocean (88, 89). The utilization and metabolism of the released organic carbon are then mainly carried out by heterotrophic microbes. Marine heterotrophic bacteria can incorporate organic carbon into biomass, respire it into CO_2, or degrade the labile fractions into refractory compounds by successive utilization (3). *Synechococcus* has also frequently been detected in deep-sea sediment traps and was estimated to contribute significant amounts of the total sediment trap POC flux (90–93). Aggregates of *Synechococcus* and heterotrophic bacteria might facilitate the sinking process. Understanding the processes of organic carbon excretion and utilization is complicated by the interactions between phototrophs and heterotrophs and how this affects global biogeochemical cycling.

In the global ocean, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* are cosmopolitan and abundant bacterioplankton populations (49, 94). In the *Synechococcus* cultures, the core and dominant microbial assemblies also contained these three bacterial clades. Is this a coincidence as a result of their abundance, or do they have some complementary metabolic characteristics and synergistic effects to sustain the ecosystem? Further metagenomic and metatranscriptomic data would shed new light on understanding the interactions between photoautotrophs and associated heterotrophic bacteria and community assembly principles relevant to natural microbial communities.

MATERIALS AND METHODS

***Synechococcus* strain isolations.** Water samples were collected from the coastal region near Xiamen (~24°N, ~118°E) and near Dalian (~38°N, ~121°E) and from the South China Sea near Yongxing Island (~17°N, ~112° E) in April 2014. The coastal area near Xiamen is a typical tropical or subtropical eutrophic oceanic region (Table S5), whereas the water near Yongxing Island is oligotrophic (Table S5). Seawater samples from the subsurface (1 m) were filtered through 3.0-μm-pore-size polycarbonate filters by gravity and the filtrates used for isolations.

Synechococcus was isolated using SN medium (95) with reduced salinity (25 ppt) for the coastal Xiamen samples. The top agar overlay method was applied to improve the efficiency of isolation (96). The bottom layer agar concentration was 1.0% (wt/vol), and the top layer concentration was 0.5%. The culture plates were incubated at 25°C under constant cool white fluorescent light (Philips, Somerset, NJ, USA) at 10 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$. *Synechococcus* colonies usually appeared after 1 to 2 months of incubation. Colonies with different appearances or morphologies were picked and inoculated to fresh plates for further purification. Single colonies (after 2 to 3 rounds of purification) were then inoculated into 96-well microtiter plates (Corning, NY, USA), with each well containing 200 μl of SN medium. The cultures were then scaled up in steps (10 ml, 100 ml, and 500 ml). *Synechococcus* strain Cy04 was isolated independently from the coastal region near Dalian with the same method described above. *Synechococcus* isolations from the South China Sea samples used the same incubation conditions as described above but were done in PRO2 liquid medium (21), with the water samples diluted until a single *Synechococcus* internal transcribed spacer (ITS) sequence (after ~4 to 7-rounds of dilutions) was obtained.

The scaled-up *Synechococcus* sp. XM-24 culture was incubated at 25°C at 10 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ without shaking (to reduce the risk of external contamination). However, the culture was mixed well in the laminar flow work area before each sampling. Three replicates that were inoculated by different growth phases of *Synechococcus* liquid cultures were performed to detect the dynamic patterns of *Synechococcus* and associated heterotrophic bacteria with growth.

Electron microscopy. Cell morphologies were examined by transmission electron microscopy (JEM-1230; JEOL), using negative staining with phosphotungstic acid on cells collected in the stationary phase of growth.

Cell collections, DNA extractions, and PCR amplification of *Synechococcus* ITS regions. For extractions of total culture DNA from the 10 different environmental isolates, 6 to 8 ml of liquid *Synechococcus* culture (in the stationary or decline phase) was collected and centrifuged. For collection of size-fractionated samples, 6 to 8 ml of the *Synechococcus* liquid culture was sequentially filtered through 3- and 0.22-μm-pore-size polycarbonate filters (47-mm diameter; Millipore) at a pressure of $<$0.03 MPa. To remove free-living cells from the 3-μm filters, a second clean 3-μm filter was placed on top of the first one with cells, and the filters were turned over. Autoclaved 0.22-μm-filtered seawater was then used to wash the cells located between the two filters and the two filters were retained to represent the $>$3.0-μm fraction. Cell pellets and filters were flash-frozen in liquid nitrogen and then stored at $-$80°C until DNA extraction.

Nucleic acids were extracted with hot sodium dodecyl sulfate, phenol, chloroform, and isoamyl alcohol (97). The quality and quantity of DNA were assessed using a NanoDrop device (ND-2000; Thermo Fisher). The DNA was stored at $-$80°C until use.

Synechococcus ITS DNA fragments were amplified using the 16S-1247f and 23S-241r primer set (98). The PCR mixtures (50 μl total volume) contained 5 μl of 10$\times$ *Taq* polymerase buffer, 0.2 mM each

dinucleoside triphosphate (dNTP), 0.5 μM each primer, 1.25 U of *Taq* DNA polymerase (TaKaRa, Japan), and 20 to 200 ng of template DNA.

TOC and DOC sampling and measurement. For total organic carbon (TOC) sampling, 20-ml samples were collected directly into 40-ml glass vials (CNW, Germany) and immediately stored at −20°C for further analysis. For DOC sampling, 20-ml samples were filtered through precombusted (450°C for 4 h) 0.7-μm-pore-size GF/F filters (47-mm diameter; Whatman, Maidstone, UK) into 40-ml glass vials and stored at −20°C after sampling. To prevent carbon contamination, all glass materials used for sample collection and storage were acid-washed, rinsed with Milli-Q (ion- and nuclease-free) water, and precombusted, as described above. TOC and DOC concentrations were measured by high-temperature catalytic oxidation (HTCO) using a Shimadzu TOC-VCPH analyzer. Before analysis, samples were melted and acidified to a pH of ≈2 with phosphoric acid. POC concentrations were obtained by subtracting the DOC concentrations from the TOC concentrations. The *Synechococcus* culture with original inoculum ratio of *Synechococcus* to heterotrophic bacteria (1.77×10^7 to 2.52×10^7 cells · ml^{-1}, described in Fig. 2A) was chosen to be representative of the analyses of organic carbon concentrations.

Counts of *Synechococcus* and heterotrophic bacterial cells. *Synechococcus* and heterotrophic bacteria cell numbers were measured using flow cytometry, as described previously (99), in *Synechococcus* sp. XM-24 culture. Briefly, an Epics Altra II flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) equipped with an external quantitative sample injector (Harvard Apparatus PHD 2000; Instech Laboratories, Inc.) was used to quantify *Synechococcus* and the total bacterial cells. For *Synechococcus*, they were identified from plots of side scatter versus red fluorescence and orange fluorescence versus red fluorescence (100). Total bacteria were enumerated according to the method of Marie et al. (101) in plots of red fluorescence versus green fluorescence and side scatter versus green fluorescence after staining with SYBR green I. Subtracting the *Synechococcus* count from the total bacteria count gave the number of heterotrophic bacteria. All flow cytometry data were analyzed using the Expo 32 Multi-COMP software (Beckman Coulter, Inc.).

Sequence generation and processing. DNA extractions from *Synechococcus* culture samples, without size fractionation, were used for amplification of the bacterial hypervariable V4 region of 16S rRNA gene using the primers 520F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) (102). Sequencing libraries were constructed using the NEBNext Ultra DNA library prep kit for Illumina (New England BioLabs, USA), according to the manufacturer's recommendations in terms of NEBNext end prep, adaptor ligation, size selection, or cleanup of adaptor-ligated DNA, PCR enrichment of adaptor-ligated DNA, and cleanup of PCR amplification. The library quality was assessed with a Qubit 2.0 fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on the Illumina MiSeq (Illumina, San Diego, CA, USA) platform. The low-quality reads (*k*-mer 5 < Q20, length < 150 bp, or with ambiguous base calls) were removed, and the paired-end 250-bp reads were then combined using the FLASH software version 1.2.7 (103). Questionable reads, with homopolymer length of >8 bases or a 5′ primer mismatch of >1, were removed from the total read pool by QIIME version 1.8.0 (104). Chimeras were removed by USEARCH version 5.2.236 (105), and UCLUST was employed to cluster the remaining high-quality reads to generate operational taxonomic units (OTUs) with an identity cutoff of 0.97 (106). The OTUs were taxonomically classified based on the Greengenes release 13.8 (107) within the QIIME pipeline, with default parameters (104). OTUs with a relative abundance of <0.001% were removed to avoid inflated estimates of diversity (108).

For the 58 size-fractionated samples from *Synechococcus* strain XM-24 cultures, the sequence manipulations were similar to those described above, but different primers, targeting the bacterial V3-V4 region (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′; and 907R, 5′-CCGTCAATTCMTTTRAGTTT-3′) of the 16S rRNA gene (109, 110), were used. With changes in sequencing technology, the read length (with paired-end 450-bp reads) that could be sequenced by Illumina greatly increased. Therefore, the 16S rRNA primers covering a longer region were used for the subsequent 58 samples.

Accession number(s). The OTU sequence data were deposited in the NCBI Sequence Read Archive under BioProject no. PRJNA378717. The sequences of the size-fractionated samples were deposited in the NCBI database with BioProject no. PRJNA362761. The ITS sequences of 10 *Synechococcus* strains have been submitted to the GenBank databases with accession numbers KY930773 to KY930776 and KY933643 to KY933648.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01517-17.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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We declare no competing interests.

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