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Comparison of the Seasonal Variations of *Synechococcus* Assemblage Structures in Estuarine Waters and Coastal Waters of Hong Kong

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Seasonal variation in the phylogenetic composition of *Synechococcus* assemblages in estuarine and coastal waters of Hong Kong was examined through pyrosequencing of the *rpoC1* gene. Sixteen samples were collected in 2009 from two stations representing estuarine and ocean-influenced coastal waters, respectively. *Synechococcus* abundance in coastal waters gradually increased from 3.6×10^3 cells ml^{-1} in March, reaching a peak value of 5.7×10^5 cells ml^{-1} in July, and then gradually decreased to 9.3×10^3 cells ml^{-1} in December. The changes in *Synechococcus* abundance in estuarine waters followed a pattern similar to that in coastal waters, whereas its composition shifted from being dominated by phycoerythrin-rich (PE-type) strains in winter to phycocyanin-only (PC-type) strains in summer owing to the increase in freshwater discharge from the Pearl River and higher water temperature. The high abundance of PC-type *Synechococcus* was composed of subcluster 5.2 marine *Synechococcus*, freshwater *Synechococcus* (F-PC), and *Cyanobium*. The *Synechococcus* assemblage in the coastal waters, on the other hand, was dominated by marine PE-type *Synechococcus*, with subcluster 5.1 clades II and VI as the major lineages from April to September, when the summer monsoon prevailed. Besides these two clades, clade III cooccurred with clade V at relatively high abundance in summer. During winter, the *Synechococcus* assemblage compositions at the two sites were similar and were dominated by subcluster 5.1 clades II and IX and an undescribed clade (represented by *Synechococcus* sp. strain miyav). Clade IX *Synechococcus* was a relatively ubiquitous PE-type *Synechococcus* found at both sites, and our study demonstrates that some strains of the clade have the ability to deal with large variation of salinity in subtropical estuarine environments. Our study suggests that changes in seawater temperature and salinity caused by the seasonal variation of monsoonal forcing are two major determinants of the community composition and abundance of *Synechococcus* assemblages in Hong Kong waters.

Members of the *Synechococcus* group of widely distributed and abundant picocyanobacteria are important primary producers in the surface waters of global oceans (1). Strains of *Synechococcus* are both phenotypically and phylogenetically diverse and dynamic (2, 3). Based on gene markers, like the 16S rRNA gene, marine *Synechococcus* strains form a well-defined clade termed cluster 5 (4, 5), which is divided into 3 subclusters: 5.1, 5.2, and 5.3. Of these, subcluster 5.1 is the most abundant and diverse subcluster in marine environments and is further divided into at least 9 clades (6). The high genetic diversity of *Synechococcus* is reflected in the ecogeographic and temporal distribution of different ecotypes. Subcluster 5.1 clade I mainly dominates in temperate mesotrophic ocean waters, while clade II is mainly present in offshore, continental shelf, and oligotrophic warm waters (4, 7). Subcluster 5.2 *Synechococcus* strains are phycocyanin-enriched (PC-type) euryhaline strains widely distributed in coastal and estuarine waters (6). Chen et al. suggested that members of this subcluster exhibit higher genetic diversity than subcluster 5.1 *Synechococcus* due to their complex habitats (8). Subcluster 5.3 *Synechococcus* strains are less studied, phycoerythrin-enriched (PE-type) strains (pigment types 3b and 3d) (http://roscoff-culture-collection.org/strains/shortlists/taxonomic-groups/marine-synechococcus) and include at least six clades (9).

In addition to the inherent genetic diversity, environmental factors also determine the variations in *Synechococcus* abundance and diversity in a particular environment (10–15). Temperature, for example, is one of the most important parameters determining the abundance of *Synechococcus* (10, 16, 17). However, to date, only a few studies have reported the temporal changes in the *Synechococcus* assemblage structure in detail (4, 18, 19).

Hong Kong coastal waters have a unique and highly dynamic hydrographic setting and therefore are ideal sites to study the diversity and spatiotemporal variation in the *Synechococcus* community composition (20). This region is influenced by several different water masses, including the freshwater discharge from the Pearl River, oceanic water of the South China Sea, and the Fujian-Zhejiang coastal current. The relative strengths and interactions of these water masses are modulated by the annual cycle of the monsoonal wind. A recent study found that estuarine waters of the Pearl River were dominated by PC-type *Synechococcus*, while the coastal waters on the other side of Hong Kong were dominated by PE-type *Synechococcus* (21). However, the phylogenetic composition of PE- and PC-type *Synechococcus* strains in Hong Kong waters remains unknown. Therefore, the present study was conducted to reveal the seasonal variation of the *Synechococcus* assemblage composition in coastal and estuarine wa-

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ters of Hong Kong, particularly in response to the changes in the Pearl River plume. PCR-based molecular methods have been successfully employed to examine the community structure of *Synechococcus* in marine waters (7, 22). In addition to the 16S rRNA gene, gene markers with even higher genetic resolution, such as the rRNA internally transcribed spacer (ITS), *narB*, *ntcA*, and *rpoC1*, are now being used to study the community structure of *Synechococcus* (22, 23). In fact, *rpoC1* was the first gene marker to show the presence of different *Synechococcus* clades in seawater samples (24), which seemed to be a promising approach.

In this study, we applied pyrosequencing of the *rpoC1* gene to investigate *Synechococcus* assemblage compositions in Hong Kong waters. Because of their sequencing depth, pyrosequencing methods reveal information on both dominant members and rare species (25, 26). We documented high diversity of *Synechococcus* strains in Hong Kong waters and recorded spatiotemporal variation of *Synechococcus* assemblage compositions. Both the phenotypic and phylogenetic compositions of *Synechococcus* assemblages are markedly influenced by river inputs.

MATERIALS AND METHODS

Sample collection. Field sampling was conducted at two Hong Kong marine stations, NM3 (113°56.7′E, 22°21.3′N) and PM7 (114°17.7′E, 22°20.4′N), with different hydrographic and trophic conditions (Fig. 1). NM3, located in the Pearl River estuary, represented estuarine waters, while PM7, at Port Shelter in the eastern waters of Hong Kong, represented ocean-influenced coastal water (here termed coastal waters). Surface water samples were collected from the two stations every month from March to December 2009, except in August and November.

At each station, 0.5 liter of surface seawater was prefiltered through a 3.0-μm (47-mm) polycarbonate membrane (Pall Corporation) and then filtered onto a 0.22-μm (47-mm) polycarbonate membrane. Samples were frozen at −80°C immediately after filtration. The temperature and salinity of the seawater were measured using a YSI 6600 (YSIs, USA). Samples for nutrient and chlorophyll *a* (Chl *a*) analyses were collected from the two stations each month. Total inorganic nitrogen (TIN) and phosphate concentrations were measured using a Skalar Nutrient Analyzer (ThermoFisher, USA). The Chl *a* concentration was measured using a Turner Designs (Sunnyvale, CA) fluorometer after extraction with 90% acetone (27).

DNA extraction, PCR, and sequencing. DNA was extracted using the enzyme/phenol-chloroform protocol (28). Extracted DNA was eluted in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and kept at −20°C until further use. The PCR followed the protocol of Mühling et al. (23). The nested-PCR primers are highly specific for *Synechococcus* species and therefore are suitable for amplifying the *rpoC1* gene from low-DNA-concentration templates (23). The first round of PCR used the primer *rpoC1*-N5 and the C-terminal primer *rpoC1*-C, and the PCR products were used as templates for the second-round PCR with the modified primers *rpoC1*-39F (5′-ccatctcatccctgcgtgtctccgactcagnnnnnnnnnnGGNATYGTYTGYGAGCGYTG) and *rpoC1*-462R (5′-cctatcccctgtgtgccttggcagtctcagCGYAGRCFCTTGRTCAGCTT) (23). (Sequences in lowercase letters are 454 adapters for binding used in pyrosequencing, and the "n" repeat represents 10-nt barcode sequences used to identify samples. The sequences in uppercase letters are targeted to the *rpoC1* gene.) The PCR products were gel purified using a Qiaquick gel purification kit (Qiagen, Hilgen, Germany) as described by the manufacturer. Library quantification was done by fluorometry using the Quant-iT picoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen, USA). The amplicons were mixed in equal amounts and sequenced in a two-region 454 run on a GS PicoTiterPlate using a GS Junior pyrosequencing system (Roche, 454 Life Sciences, Branford, CT, USA) according to the manufacturer's instructions.

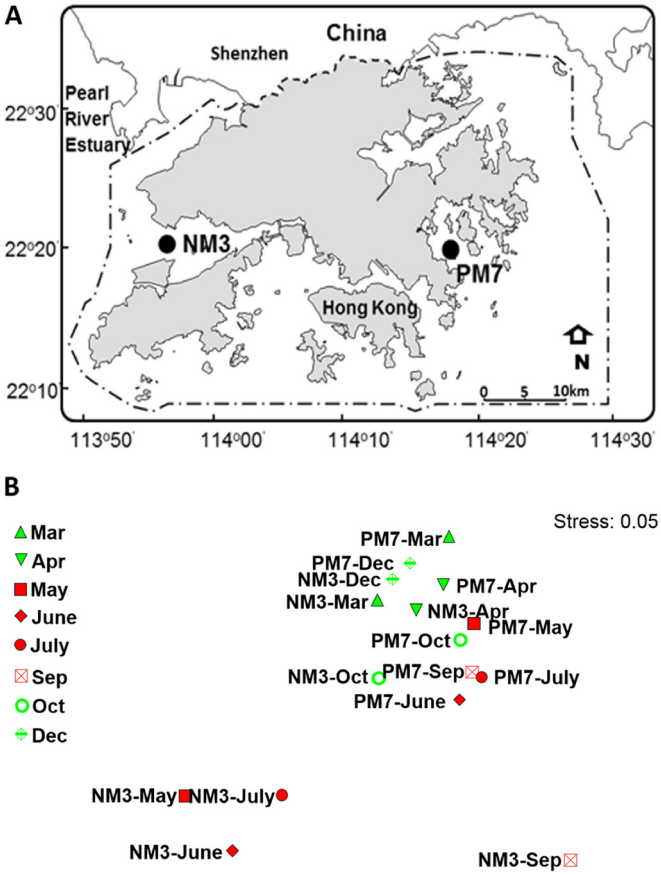

FIG 1 (A) Sampling stations. NM3 is located in the western waters of Hong Kong, which are influenced by freshwater discharge from the Pearl River, whereas PM7 is a typical coastal station located in the eastern waters of Hong Kong. (Adapted from reference 21 with permission of the publisher [copyright 2013 Society for Applied Microbiology and John Wiley & Sons Ltd.].) (B) NMDS plot showing the relationship of hydrographical and trophic conditions at sampling stations.

Postrun sequence analyses. Analysis of *rpoC1* sequence was conducted using the microbial ecology community software program mothur (http://www.mothur.org/wiki/Download_mothur) (29). The reads were processed by removing tags and primers; only reads with an average quality score above 20 and read lengths between 300 bp and 500 bp were accepted. Sequence denoise was carried out using the command shhh.seqs with sigma value 0.01. Chimeras were analyzed in the mothur software package using the command chimera.uchime (30). After the above-described quality control, 6,247 high-quality sequences were randomly subsampled from each sample and were further analyzed with mothur for alignment and DNA distance calculations.

The richness estimator (ACE), diversity index (Shannon index; *H*′), coverage, and operational taxonomic units (OTUs) were calculated at 95% similarity using mothur's summary. single routine. Rarefaction curves were calculated using ACE richness index data and the number of observed OTUs.

The representative sequence of each OTU was extracted and identified by local BLAST search using Bio-Edit (31), and the sequences used as references are listed in Table S1 in the supplemental material. Sequences that were less than 90% identical to the reference sequences were assigned as unclassified. The expectation value cutoff used was 0.01. The relative abundance of each lineage was summarized and used to calculate the abundance of *Synechococcus* lineages in each month (abundance of each lineage = relative abundance × total *Synechococcus* abundance measured by flow cytometry).

TABLE 1 Environmental parameters of sample stations

Sample	Temp (°C)	TIN (μmol/liter)	Phosphate (μmol/liter)	Salinity (ppt)	Chl *a* (mg/m^3)
PM7-Mar	18.95	1.81	0.41	34.49	4.31
PM7-Apr	22.01	1.29	0.39	34.78	2.88
PM7-May	27.30	3.15	0.47	35.52	0.78
PM7-June	27.70	7.31	0.44	29.47	7.13
PM7-July	29.70	2.63	0.45	30.84	2.27
PM7-Sep	28.90	6.71	0.46	31.45	4.27
PM7-Oct	27.60	3.05	0.55	33.30	0.99
PM7-Dec	19.80	10.45	0.61	33.87	2.74
NM3-Mar	21.00	24.33	0.60	29.92	0.70
NM3-Apr	22.90	16.69	0.48	31.70	2.76
NM3-May	26.30	78.28	1.97	22.84	2.83
NM3-June	29.00	64.37	1.84	18.41	5.17
NM3-July	28.30	35.67	1.64	19.94	3.13
NM3-Sep	29.40	14.27	0.63	28.51	19.60
NM3-Oct	27.60	13.22	1.30	32.30	0.85
NM3-Dec	20.60	9.94	0.95	33.93	2.05

Heat maps showing the relative abundances and relationships of the top 50 most abundant OTUs were generated using HemI (32). The relative abundance of each OTU was square root transformed. Average linkage clustering was performed by using Pearson correlation matrices. The representative sequence of each OTU was classified using the database mentioned above. BLASTn was also applied to sequence identity analysis (http://www.ncbi.nlm.nih.gov/).

Phylogenetic trees of PC-type *Synechococcus* sequences were constructed in MEGA 5 using the maximum-likelihood method (ML) with a Kimura 2-parameter model (33). The initial tree for ML was based on BIONJ (64). The nearest relatives were retrieved from National Center for Biotechnology Information (NCBI) reference sequences. A heat map showing the relative abundance of each OTU was generated in iTol (34). Nonmetric multidimensional scaling (NMDS) analysis, the analysis of molecular variance (AMOVA) test, and similarity percentage (SIMPER) analysis were applied using Primer 5 (Primer-E-Ltd., United Kingdom) to compare the community compositions based on the relative abundances of lineages identified from Hong Kong waters.

The relationship between environmental parameters and *Synechococcus* community composition was studied by redundancy analysis (RDA) using CANOCO V4.5 (Microcomputer Power, USA). A matrix was generated using the relative abundance of each lineage transformed by square root transformation. Environmental data were normalized using Z-score transformation. Centering and normalizing options were employed, producing scores centered and standardized to unit variance. The significance of the eigenvalues and species-environment correlations of the first three axes were determined by Monte Carlo tests (500 permutations).

Salinity tolerance of a clade IX *Synechococcus* strain in Hong Kong waters. In order to study the salinity tolerance of *Synechococcus* lineages, we evaluated the growth rates of 6 strains—WH 8012 (clade II), WH 7803 (clade V), PS01 (clade V), MW03 (clade VIII), MW02 (clade IX), and WH 5701 (subcluster 5.2)—at various salinities. MW02 and MW03 were isolated from estuarine waters of Hong Kong. The ITS gene sequence of MW02 shows it is a clade IX *Synechococcus* strain related to *Synechococcus* sp. strain RS9901 (BLASTN). PS01 is a low-phycourobilin (PUB) *Synechococcus* strain, while MW02 and MW03 are PE-only and PC-only *Synechococcus* strains, respectively. All the cultures were grown in f/2 medium enriched artificial seawater (65) (with 50 μM ammonium as an N source) with different salinities (15, 22, 28, and 34 ppt) for 15 days in plant growth chambers at 25°C under a photon flux density of 2.5×10^{15} quanta s^{-1} cm^{-2} in a 12-h/12-h light-dark cycle. The optical density at 440 nm (OD_{440}) of each culture was measured every day. Growth rates (day^{-1}) were calculated as $\ln (N_t/N_0)/dt$. N_0 and N_t are the OD_{440} values of strains at the beginning and at the end of the culture period, and dt is the culture period (in days).

***In vivo* absorption spectra.** The *in vivo* absorption spectra of the *Synechococcus* cultures were measured to determine the optical properties of *Synechococcus* strains (2, 35). An aliquot of the exponentially growing culture was transferred to a cuvette, and the *in vivo* absorption spectrum was measured from 400 to 700 nm using a spectrophotometer (UH5300; Hitachi, USA). The scan rate was 1 nm s^{-1}. The spectra were normalized at 440 nm.

Accession numbers. The ITS gene sequence of MW02 has been submitted to NCBI with accession number KP113680. All 200,079 raw sequences obtained from this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SRR1583674 (PM7-Mar), SRR1106828 (PM7-Apr), SRR1583675 (PM7-May), SRR1583673 (PM7-June), SRR2242694 (PM7-July), SRR1583676 (PM7-Sep), SRR2242696 (PM7-Oct), SRR1107754 (PM7-Dec), SRR1583670 (NM3-Mar), SRR1107750 (NM3-Apr), SRR1583671 (NM3-May), SRR1583669 (NM3-June), SRR2242693 (NM3-July), SRR1583672 (NM3-Sep), SRR2242695 (NM3-Oct), and SRR1107753 (NM3-Dec).

RESULTS

Environmental conditions at the sampling stations. The estuarine station (NM3) is strongly influenced by freshwater discharge from the Pearl River, particularly during the wet season (April to September; 80% of the discharge of the Pearl River occurs during this period [36]), making it a low-salinity and high-nutrient environment. On the other hand, the coastal station (PM7) located on the east side of Hong Kong did not receive direct river input and developed stratified conditions with nutrient depletion in the upper mixed layer in the summer (37). At PM7, the highest TIN and phosphate concentrations occurred in December because of the nutrient-rich Fujian-Zhejiang coastal current water brought to the region under the northeast monsoon (Table 1). We found that the two stations had very different hydrographic and trophic conditions during the period from May to September (Table 1 and Fig. 1B). The highest dissimilarity of environmental conditions between the two stations (90.9%) was found in May, while the lowest (16.4%) was found in December.

Sequencing statistics. A total 200,079 *rpoC1* raw sequences were obtained from 16 samples. After removing the noise, poor-quality reads, and chimera sequences, 130,739 high-quality sequences were obtained. Following random subsampling, 6,247 high-quality sequences from each sample were obtained and used for further analysis (see Table S2 in the supplemental material).

Rarefaction analysis was applied to evaluate whether screening of 6,247 sequences was sufficient to estimate the diversity of *Synechococcus* communities (see Fig. S1 in the supplemental material). The rarefaction curves of the observed OTUs did not saturate biodiversity, while some ACE index curves were close to stabilization. The coverage value of *Synechococcus* assemblages ranged from 0.825 to 0.965. The lowest value was detected in the sample PM7-July (see Table S2 in the supplemental material).

Spatiotemporal variation of *Synechococcus* lineages. In the coastal waters, *Synechococcus* abundance gradually increased from 3.6×10^3 cells ml^{-1} in March to the maximum value of 5.7×10^5 cells ml^{-1} in July and then gradually decreased to 9.3×10^3 cells ml^{-1} in December. The *Synechococcus* assemblage in coastal waters was dominated by PE-type *Synechococcus* (Fig. 2A). Changes of *Synechococcus* abundance in estuarine waters followed a pattern similar to that in coastal waters, but the community was dominated by PC-type *Synechococcus* (Fig. 2B).

Based on our database, more than 98% of our sequences were classified. All three marine *Synechococcus* subclusters (subclusters 5.1, 5.2, and 5.3) were detected at both stations. The most abundant subclusters in the coastal waters and the estuarine waters were subclusters 5.1 and 5.2, respectively (Fig. 2C and D). In total, 15 clades of subcluster 5.1 *Synechococcus* were detected in Hong Kong waters. Subcluster 5.3 *Synechococcus* sequences were only a minor component at both stations, accounting for only 1.53% and 0.96% of the total sequences in coastal waters and estuarine waters, respectively. Besides marine *Synechococcus*, *Cyanobium* and freshwater PC-type *Synechococcus* (F-PC) were also detected (Fig. 2C and D).

At the coastal station, the most abundant lineage was clade II, followed by clades VI and IX (Fig. 2E). Clade II *Synechococcus* was the core lineage that could be detected in all months. Clade VI was the second major lineage during the wet season, while clade IX was the dominant lineage in the dry season. Clade III, which cooccurred with clade V only, had high abundance in the coastal waters in July and September. Clade I was observed only in March. The undescribed clade (miyav) (22), represented by strain *Synechococcus* sp. strain miyav, mainly occurred in December and March. In July, many *Synechococcus* lineages contributed to the high abundance of *Synechococcus* in the coastal waters, including clades II, III, V, VI, WPC1, HK01, and S5.3 (Fig. 2E). In coastal waters, subcluster 5.2 *Synechococcus* could be found only occasionally, typically in summer (Fig. 2C and E).

Conversely, at the estuarine station (NM3), lineages of subcluster 5.2, F-PC, and *Cyanobium* were highly abundant during the wet season and peaked in July, likely owing to high freshwater discharge from the Pearl River (Fig. 2D). A phylogenetic tree shows that OTUs belonging to S5.2, F-PC, and *Cyanobium* are affiliated with *Synechococcus* sp. strain WH 8007, freshwater *Synechococcus* group D strains (38), and *Synechococcus* sp. strain PCC 9005 (*Cyanobium*), respectively (see Fig. S2 in the supplemental material). In comparison to the coastal waters, the diversity and abundance of subcluster 5.1 *Synechococcus* in estuarine waters were low (Fig. 2D and F). Subcluster 5.1 *Synechococcus* strains in estuarine waters were mainly contributed by clade II and clade IX; the former occurred in the dry season, while the latter occurred in all months at relatively high abundance (Fig. 2D and F). The undescribed clade (miyav) was also an important component in December (Fig. 2F). Clades IV, VII, VIII, XV, XVI, and CRD were minor lineages in Hong Kong waters. These lineages mainly occurred at NM3 in April and at PM7 in March and April (Fig. 2E and F).

At the OTU level, we found that high abundance of clade II and clade IX *Synechococcus* were comprised of multiple OTUs (Fig. 3). Clade IX OTUs were affiliated with *Synechococcus* sp. strain 59 and *Synechococcus* sp. RS9901, respectively. Unlike other clade IX OTUs, which mainly occurred in winter, OTU14 and OTU42 dominated estuarine waters in summer and clustered together with subcluster 5.2 *Synechococcus*, *Cyanobium*, and freshwater *Synechococcus* (Fig. 3).

Some OTUs showed similar niche preferences. For example, OTU10 (clade V), OTU12 (clade III), and OTU41 (WPC1) were abundant at PM7 in July and September; OTU11 (clade I), OTU19 (clade I), and OTU45 (clade CRD1) were relatively highly abundant in samples NM3-Apr and PM7-Mar.

Richness and diversity of *Synechococcus* assemblages in Hong Kong waters. The Shannon diversity index of *Synechococcus* communities at the two study sites ranged from 1.83 to 5.22. The highest *Synechococcus* community diversity and richness in coastal waters and estuarine waters occurred in July and April, respectively (Fig. 4). The diversity index of the *Synechococcus* community in coastal waters was much higher than that in estuarine waters in July, when the highest *Synechococcus* abundance was recorded at both stations (Fig. 2 and 4). The richness index, ACE, showed results similar to those of the Shannon index (Fig. 4). Even though the abundance of *Synechococcus* was low in winter, the diversity of *Synechococcus* was still high at both stations.

Similarity among *Synechococcus* assemblages in Hong Kong waters. NMDS plots showed that the compositions of *Synechococcus* assemblages at the two stations were similar in December (Dry) but differed greatly in the wet season (Fig. 5). The assemblages formed three groups, PM7(Summer), NM3(Wet), and Dry. The similarity of assemblages in each group was higher than 60%. The first group, PM7(Summer), was composed of samples collected from stratified water at PM7 when the summer monsoon prevailed. Sample PM7-June, however, deviated from this group, presumably due to the high abundance of subcluster 5.2 *Synechococcus*, which originated in the estuarine waters and was brought to the coastal waters by freshwater discharge. The second group, NM3(Wet), was composed of samples collected at NM3 from March to October, excluding those collected in April, when freshwater discharge from the Pearl River influenced the station. The last group, Dry, contains samples PM7-Dec, PM7-Oct, and NM3-Dec. The compositions of the three groups were significantly different (AMOVA; $P < 0.05$). SIMPER analysis was performed to determine each lineage's contribution to dissimilarity between the three groups. The dissimilarities were 88.2% between PM7(Summer) and NM3(Wet), 77.1% between PM7(Summer) and Dry, and 73.1% between NM3(Wet) and Dry. In summer, the difference between the assemblages in NM3 and PM7 was mainly contributed by subcluster 5.2, clade VI, clade II, and *Cyanobium*. In NM3, the variation of *Synechococcus* assemblages between summer and winter was mainly caused by an increase of subcluster 5.2 and a decrease of clade IX in summer; while in PM7, it was mainly contributed by clade IX and clade VI (Fig. 6).

Impact of environmental parameters on *Synechococcus* community compositions. The sum of all canonical eigenvalues showed that 57.0% of the observed assemblage variation could be accounted for by environmental variables. Of all the measured environmental parameters, salinity (Monte Carlo test; $P = 0.002$;

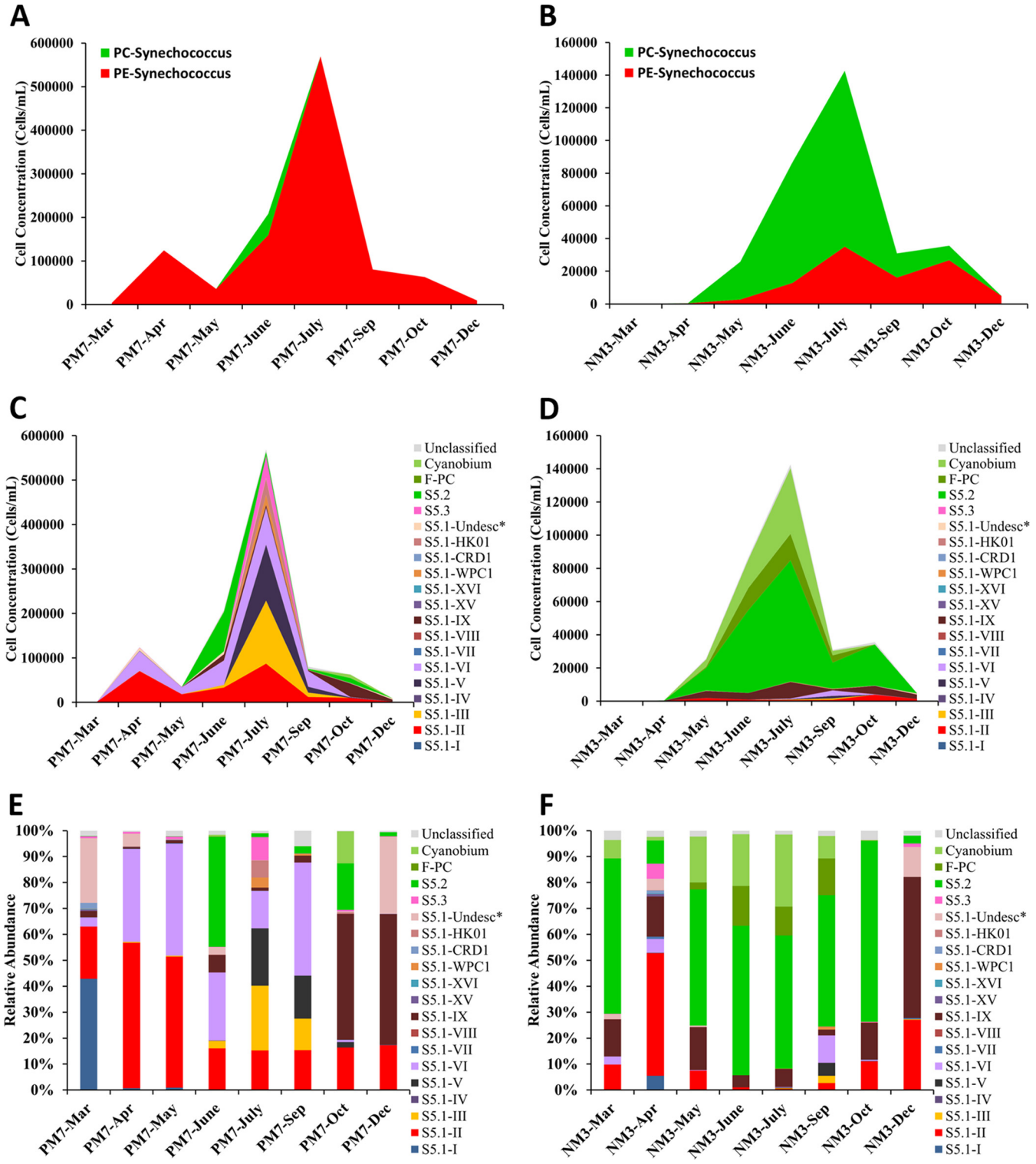

FIG 2 Abundances and relative abundances of *Synechococcus* lineages in Hong Kong waters. (A and B) Abundances of PC-type and PE-type *Synechococcus* in Hong Kong waters measured by flow cytometry analysis (replotted from Liu et al. [21]). (C and D) Calculated abundances of *Synechococcus* lineages in each month (abundance of each lineage = relative abundance × total *Synechococcus* abundance measured by flow cytometry). (E and F) Relative abundances of *Synechococcus* lineages in samples collected in each month. *, S5.1-Undesc is the undescribed clade represented by *Synechococcus* sp. strain miyav.

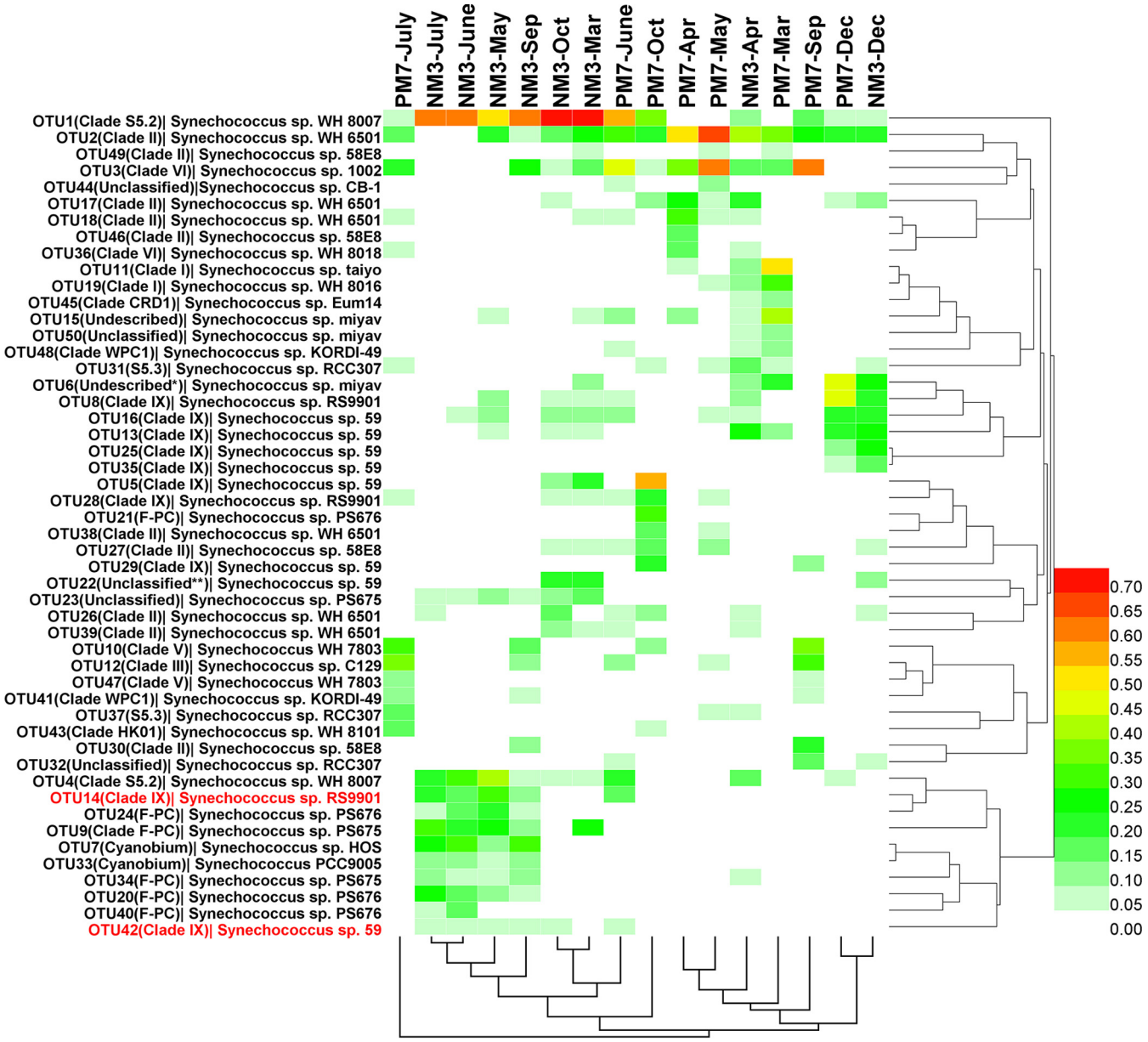

FIG 3 Heat map displaying the relative abundances of the top 50 most abundant OTUs across the samples. The data were transformed by square root transformation. The OTU name, lineage, and most similar strain in the NCBI database are shown on the left. OTU14 and OTU42 (red), affiliated with clade IX *Synechococcus* sp. RS9901, have a niche similar to that of PC-type *Synechococcus*. *, undescribed clade represented by *Synechococcus* sp. strain miyav; **, unclassified OTU (the OTU's representative sequence was less than 90% identical to the reference sequences in this study).

F ratio = 6.57) and temperature (Monte Carlo test; $P = 0.012$; F ratio = 3.72) had significant impacts and explained 32.0% and 22.0% variance of the *Synechococcus* assemblages, respectively (Fig. 7).

The abundances of *Synechococcus* clades I, IV, VII, CRD1, XV, and XVI were positively related to salinity but negatively related to Chl *a* and temperature. Clade II abundance was positively related to salinity and negatively related to TIN and phosphate. Subcluster 5.2, *Cyanobium*, and F-PC *Synechococcus* abundances were negatively related to salinity but positively related to temperature. Clade IX and the undescribed clade (represented by *Synechococcus* sp. strain miyav) were dominant in the dry season and were negatively related to temperature (Fig. 7).

Growth of *Synechococcus* strains in media with different salinities. Both PC-type *Synechococcus* strains (MW03 and WH 5701) showed high tolerance for salinity variations and grew at salinities from 15 ppt to 34 ppt. The PE-type *Synechococcus* strains WH 7803, PS01, and WH 8012 could not tolerate salinity lower than 15 ppt (Fig. 8). WH 7803 and WH 8012 showed higher growth rates at salinities of 28 ppt and 34 ppt, while PS01 had the highest growth rates (0.14 ± 0.001 day^{-1}) at 28 ppt. In comparison, PE-type *Synechococcus* sp. strain MW02 (pigment type 2) (see Fig. S3 in the supplemental material), isolated from the estuarine waters of Hong Kong, could tolerate a wide range of salinity, which is consistent with its distribution in Hong Kong waters. MW02 showed the highest growth rates (0.12 ± 0.007 day^{-1}) at 28 ppt.

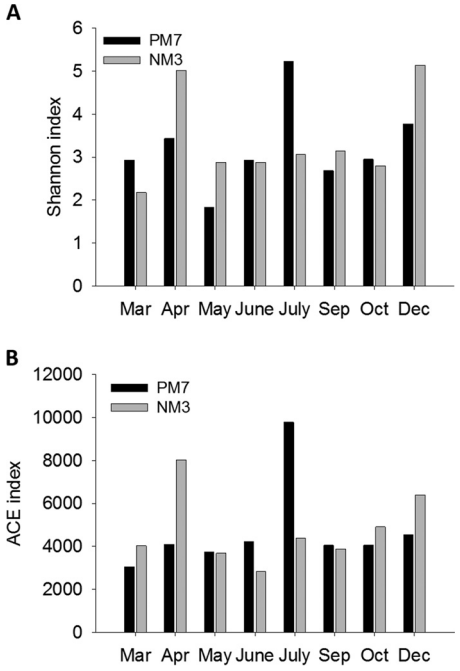

FIG 4 Diversity and richness indices of *Synechococcus* assemblages in Hong Kong waters.

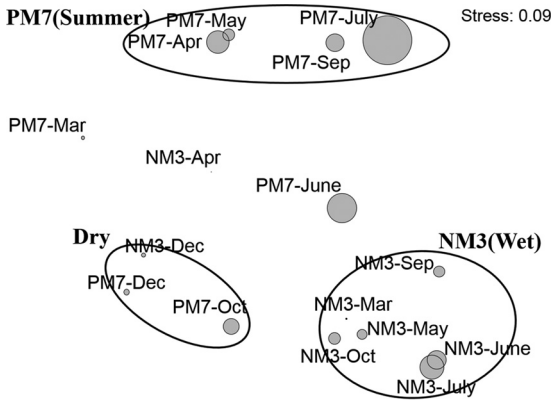

FIG 5 NMDS plot showing the relationship of the *Synechococcus* communities based on the relative abundance of each *Synechococcus* lineage. The samples formed three groups, PM7(Summer), NM3(Wet), and Dry. The AMOVA test showed that the compositions of the three groups were significantly different from each other. The sizes of the bubbles indicate the abundance of *Synechococcus* in each sample (the abundance of each sample was square root transformed).

DISCUSSION

Pyrosequencing the *rpoC1* gene from 16 samples from Hong Kong waters showed different patterns of seasonal variation in the *Synechococcus* assemblage composition in estuarine waters and coastal waters. *Synechococcus* assemblage diversity was high in high-temperature and stratified coastal waters in summer but relatively low in freshwater-influenced estuarine waters. In summer, the *Synechococcus* assemblage in estuarine waters was dominated by PC-type *Synechococcus* strains, which were composed of subcluster 5.2, freshwater *Synechococcus*, and *Cyanobium*. In contrast, the *Synechococcus* assemblage in coastal waters in summer was dominated by PE type subcluster 5.1 *Synechococcus* lineages. PE-type *Synechococcus* strains isolated from estuarine waters were highly tolerant of variations in salinity.

Sensitivity of the pyrosequencing application. Different gene markers have been used to characterize marine *Synechococcus* diversity (22, 39, 40). Most protein gene markers provide higher resolution of genetic diversity in marine *Synechococcus* than the 16S rRNA gene (4, 23). The ITS region is a widely used and accurate marker for *Synechococcus* identification, but it did not seem suitable for pyrosequencing, as the method would not cover the whole ITS gene sequence. In contrast, a 423-bp fragment of the *rpoC1* gene used for *Synechococcus* identification could be covered by the output of the 454 pyrosequencing method and is single copy in *Synechococcus* cells. In this study, all major *Synechococcus* clades were detected in Hong Kong waters by pyrosequencing of the *rpoC1* gene, which indicates that the method is suitable for evaluating marine *Synechococcus* assemblage composition and diversity.

As mentioned above, only a few studies have reported about the *Synechococcus* community composition in Hong Kong waters. The most recent one (21), which used the flow cytometry approach, reported the presence of PC-type *Synechococcus* in the coastal waters only in June, in contrast to our findings of the presence of the same clade throughout most of the year. These contradictions may have arisen due to the differences in the methods used in the two studies. We also found *Cyanobium* in estuarine waters of Hong Kong, which had not been reported in a previous study based on clone library analysis (21). Use of next-generation DNA-sequencing methods with high sensitivity essentially yields more insights into the microbial community composition, and we highlight the need for such approaches to fully discover the community composition of *Synechococcus* lineages.

Abundance and diversity of *Synechococcus* assemblages in Hong Kong waters. Temperature is an important factor that determines the abundance of *Synechococcus* (41–43). Growth of *Synechococcus* exceeds its grazing mortality in summer, resulting in a high abundance of *Synechococcus*. In winter, low growth rates caused by low temperature make the bacteria unable to keep pace with grazing, and as a result, *Synechococcus* abundance becomes low. This is similar to what we found, where *Synechococcus* abundance in Hong Kong waters is low in winter but high in summer (Fig. 2A and B). In response to increases of temperature during the wet season (April to September), *Synechococcus* cell density increased from several hundred cells ml^{-1} to more than 5.5×10^5 cells ml^{-1} in coastal waters and 1.4×10^5 cells ml^{-1} in estuarine waters in July. Low salinity, highly turbid, and high-nutrient estuarine waters mainly selected for subcluster 5.2 *Synechococcus*, while relatively oligotrophic coastal water selected for subcluster 5.1 *Synechococcus* (Fig. 2D and E). These results agree with the definition of subcluster 5.1 and 5.2 *Synechococcus* (44) and support the idea that *Synechococcus* groups can serve as indicator organisms for estuarine hydrodynamics (45).

Based on pyrosequencing of the *rpoC1* gene, we found 17 clades representing *Synechococcus* subclusters 5.1, 5.2, and 5.3 and also freshwater *Synechococcus* and *Cyanobium* species (Fig. 2). In comparison, studies of the tropical/subtropical Pacific Ocean (9), Chesapeake Bay (46), East China Sea (19), California current (18), and Sargasso Sea (47) found about 6 to 11 *Synechococcus* clades. Although 12 or 13 *Synechococcus* clades were reported in the Gulf of Aqaba by constructing *ntcA* gene clone libraries and pyrose-

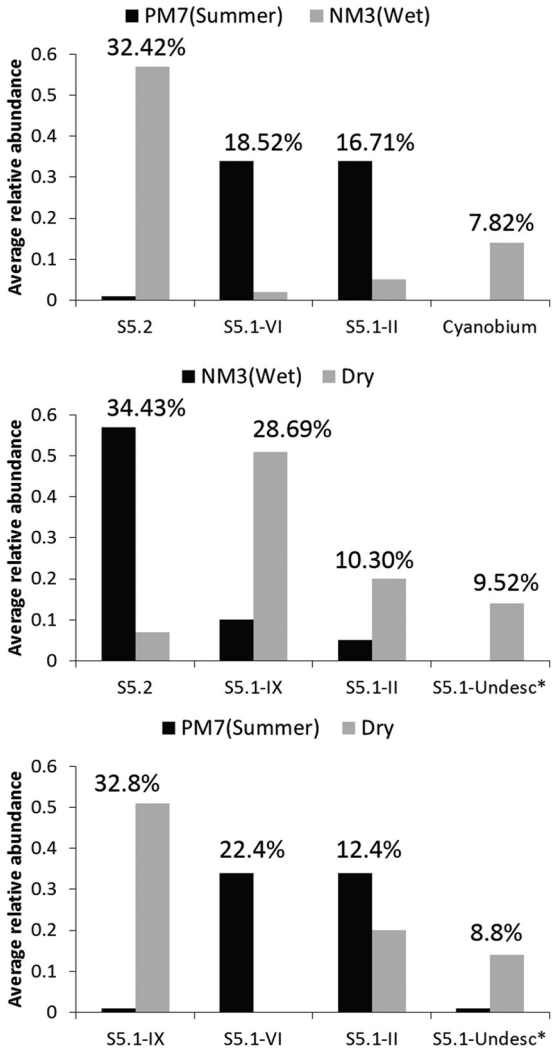

FIG 6 Four lineages that contribute most to the variation among group PM7(Summer), group NM3(Wet), and group Dry using SIMPER analysis. The bars show the average relative abundance of each lineage in each group; the numbers above the bars are the contributions of each lineage to the dissimilarity between groups. The three groups were determined according to an NMDS plot. *, undescribed clade (Undesc).

quencing of the V6 region of the 16S rRNA gene, the data were combined from several different studies conducted in multiple years (48). Our results suggest that Hong Kong coastal waters are one of the regions with the world's highest *Synechococcus* diversity.

Seasonal variation of *Synechococcus* assemblages in Hong Kong coastal waters. Seasonal variation of the *Synechococcus* assemblage composition in coastal waters was mainly caused by different subcluster 5.1 lineages. Clade II dominated the *Synechococcus* assemblages throughout the year; clade III occurred in summer, when stratification developed; and clade VI was a major lineage during the transition periods between mixing and stratification, which is in agreement with the study conducted in the Gulf of Aqaba (48). However, that study was unable to distinguish clades V and VI, and therefore, no information on the niche difference between the two clades was obtained. We found that clade V, which occurred only in highly stratified waters from July to September, had a narrower niche than clade VI, even though they cooccur in summer. Clade IX, which was first found in the Gulf of Aqaba (4), has a low abundance in the global ocean (7) and is also even a rare species in the Gulf of Aqaba (49), but it thrived in Hong Kong coastal waters during winter. It is worth noting that the diversity of the *Synechococcus* assemblage was highest in the coastal waters of Hong Kong in July (Fig. 4). Overall, our results suggest that Hong Kong coastal waters are an ideal environment for the growth of most subcluster 5.1 and 5.3 *Synechococcus* lineages when it has high temperature and is strongly influenced by the water from the South China Sea driven by the southwest monsoon. In addition to the abundant clades described above, several opportunistic *Synechococcus* groups with narrow niches were found in Hong Kong coastal waters. For example, clade I *Synechococcus* strains are typical of temperate cold waters (7) and also occur in upwelling waters (50) and mixing waters (48). They appeared in Hong Kong waters during winter mixing. Clades XV and XVI are capable of chromatic adaptation and were first isolated from the Sargasso Sea (latitude 34° to 35°N) (47). It has been suggested that these two clades are adapted to low-light conditions (50). In Hong Kong waters, they appeared in March, when strong mixing occurred. Clade III has a narrow temperature spectrum (51), and it was found in July, when the temperature was higher than 29°C. The apparently quick appearance (and disappearance) of these clades in response to changes in the environment meets the criteria for a "microbial seed bank," which suggests that microorganisms are able to enter a reversible state of low metabolic activity under harsh environmental conditions and can be resuscitated when the conditions favor growth (52).

Seasonal variation of *Synechococcus* assemblages in Hong Kong estuarine waters. Unlike in coastal waters, the increase in discharge from the Pearl River shifted the *Synechococcus* community structure in estuarine waters from PE-type subcluster 5.1 lineages to PC-type *Synechococcus* lineages. PC-type *Synechococcus* strains have greater absorbance in red light, enabling them to grow well in highly turbid, red-light-dominant estuarine waters (53). Also, they have large genomes (54), potentially to deal with complex hydrographic conditions in the estuarine environment. Moreover, it has been suggested that PC-type *Synechococcus* strains have a greater ability to deal with salinity variation than marine PE-type *Synechococcus* (4, 55). Although high abundance of PC-type *Synechococcus* strains was often found in estuarine waters (56, 57), only a few studies have reported their community composition at the molecular level. In this study, we found that PC-type *Synechococcus* assemblages in Hong Kong estuarine waters were composed of marine-source *Synechococcus* (subcluster 5.2, represented by *Synechococcus* sp. WH 8007), freshwater *Synechococcus* (F-PC), and *Cyanobium* (see Fig. S2 in the supplemental material). This result supports the hypothesis, proposed by Liu et al. (21), that the reported *Prochlorococcus*-like picocyanobacteria in fresh and brackish waters (58, 59) are likely PC-type *Synechococcus* or *Cyanobium*. Subcluster 5.2 *Synechococcus* sp. WH 8007, which was the most abundant PC-type *Synechococcus* strain in Hong Kong waters, is also the major PC-type *Synechococcus* in Chesapeake Bay (46). Similar to the result of the study in Chesapeake Bay, we also did not find *Synechococcus* strains in Hong Kong waters that were closely related to *Synechococcus* sp. strain WH 5701 (8), which is another representative strain of subcluster 5.2. This suggests that strain clusters represented by WH 5701 and WH 8007 have different niches.

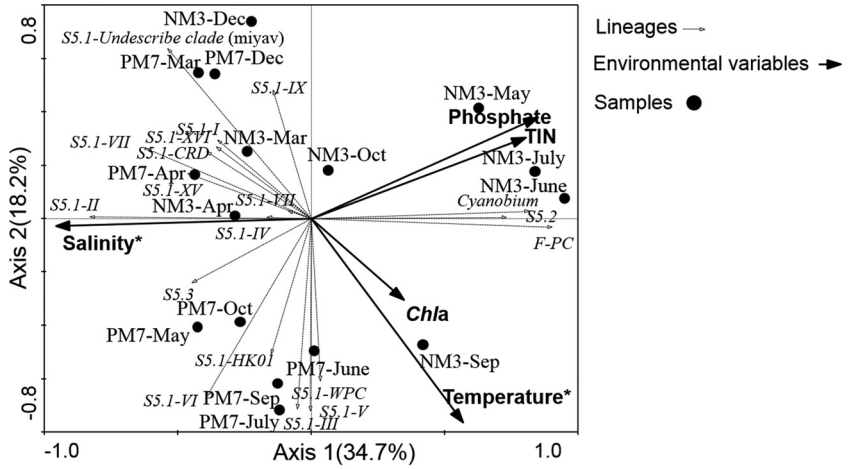

FIG 7 Correlation triplot based on an RDA depicting the relationship between the environmental factors and *Synechococcus* communities. The relative abundance of each lineage was normalized by square root transformation, and environmental data were Z-score transformed. *, factors significantly correlated with variation in the *Synechococcus* community ($P < 0.05$).

Besides the PC-type subcluster 5.2 *Synechococcus* strains, we also found that the increase in the freshwater *Synechococcus* abundance followed the increase in the discharge from the Pearl River. This implies that advection of the allochthonous *Synechococcus* population from the river inflow can be important for the estuarine ecosystem (60) during the wet season. Chen et al. reported that freshwater *Synechococcus* strains are rare in the Chesapeake Bay, where high abundance of PC-type *Synechococcus* was detected (46). In contrast, freshwater *Synechococcus* was found in Hong Kong waters, based on sequencing of the *cpcBA* gene (21). Consistently, more than 1.539×10^4 cells ml^{-1} of freshwater *Synechococcus* strains were detected in estuarine waters in July in this study (Fig. 2B). We further revealed that freshwater *Synechococcus* strains in Hong Kong estuarine waters were affiliated with PC-

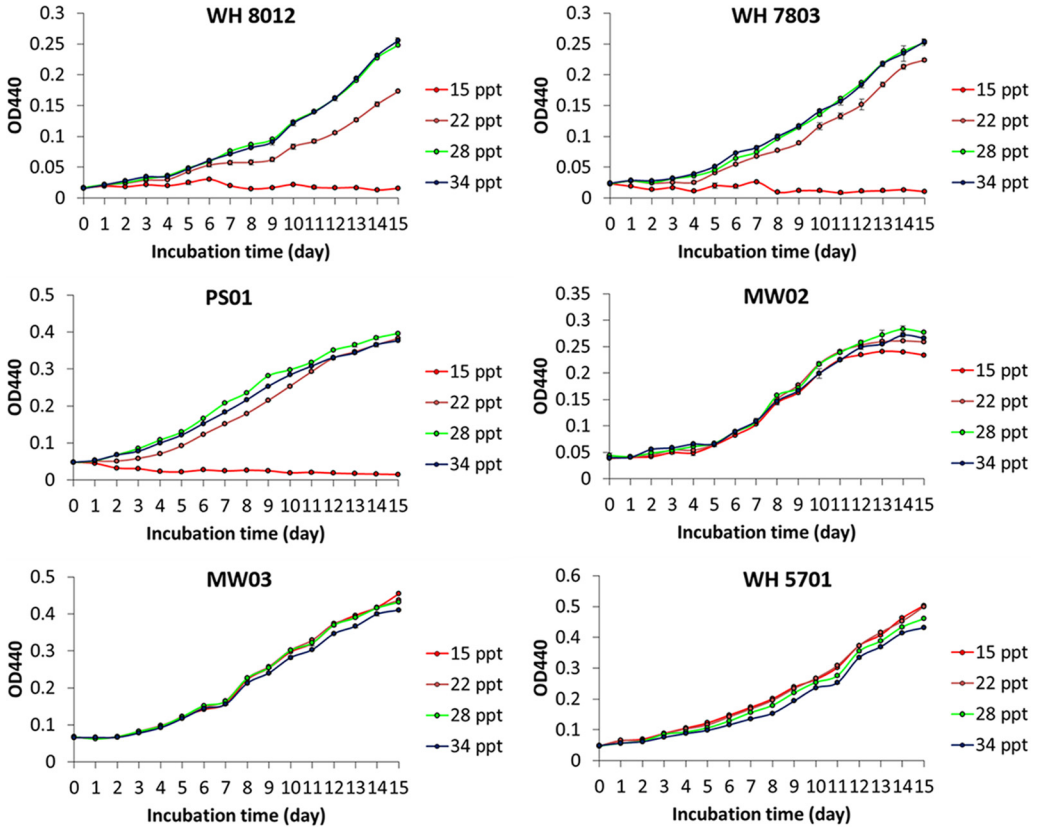

FIG 8 Growth curves of *Synechococcus* strains in f/2 media with different salinities. MW03 and WH 5701 are PC-type *Synechococcus*. WH 8012, WH 7803, PS01, and MW02 are PE-type *Synechococcus*.

type *Synechococcus* sp. strain PS676, which was isolated from Lake Teganuma (Japan). The presence of a high abundance of freshwater *Synechococcus* strains in Hong Kong estuarine waters suggests freshwater *Synechococcus* strains are abundant in the Pearl River.

Cyanobium mainly occurs in freshwater and brackish environments and was also detected in estuarine waters of Hong Kong during May to September, when the discharge from the Pearl River was high. Many studies have reported that the taxonomic distinction between the *Cyanobium* and *Synechococcus* taxa is poorly defined (61, 62). Indeed, phylogenetic analysis shows that the *Cyanobium* strains found in this study were most closely related to *Synechococcus* sp. PCC9005 (*Cyanobium*) and formed a cluster with *Synechococcus* sp. WH 5701 (see Fig. S2 in the supplemental material).

PE-type *Synechococcus* in estuarine waters in summer. Subcluster 5.1 *Synechococcus* strains have been defined as strictly marine strains that are unable to grow well in low-salinity environments (44). For example, PE-type *Synechococcus* sp. strain WH 7803 and strain WH 7805 cannot grow well when the salinity is less than 28 ppt (55). However, recently, Chen et al. also showed that PE-type *Synechococcus* sp. strain CB0205 and strain CB0208, isolated from the Chesapeake Bay, are able to grow in SN medium with a wide range of salinities (8). In this study, we noticed that subcluster 5.1 clade IX *Synechococcus* sp. MW02, isolated from Hong Kong waters, could also likely survive in low-salinity waters based on pyrosequencing data (Fig. 2), and this was confirmed by a growth experiment (Fig. 8). Either this is a characteristic of all clade IX strains or it might be that some *Synechococcus* clades have different subclades adapted to different environments (Fig. 3). This result may also support the finding of Junier et al. (63) that some freshwater *Synechococcus* strains are closely related to clade IX *Synechococcus* sp. RS9901.

Conclusions. Using a high-throughput sequencing method, we found an unprecedentedly high phylogenetic diversity of *Synechococcus* strains, an important unicellular cyanobacterial primary producer, in subtropical waters of Hong Kong. Temperature and salinity are the main factors influencing the *Synechococcus* community composition in Hong Kong waters. Furthermore, the variation in freshwater discharge from the Pearl River strongly influences the abundance and composition of the *Synechococcus* community in estuarine waters. Unlike most PE-type *Synechococcus* strains, which cannot survive in low-salinity estuarine waters, we found that some clade IX *Synechococcus* strains that are common in our study sites can grow at a range of salinities. Further research on the genome of euryhaline PE-type *Synechococcus* strains, especially clade IX, may help us to better understand the mechanism by which these *Synechococcus* cells respond to salinity changes.

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