

Genetic Diversity and Temporal Variation in the Cyanophage Community Infecting Marine *Synechococcus* Species in Rhode Island's Coastal Waters

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The cyanophage community in Rhode Island's coastal waters is genetically diverse and dynamic. Cyanophage abundance ranged from over 10^4 phage ml^{-1} in the summer months to less than 10^2 phage ml^{-1} during the winter months. Thirty-six distinct cyanomyovirus g20 genotypes were identified over a 3-year sampling period; however, only one to nine g20 genotypes were detected at any one sampling date. Phylogenetic analyses of g20 sequences revealed that the Rhode Island cyanomyoviral isolates fall into three main clades and are closely related to other known viral isolates of *Synechococcus* spp. Extinction dilution enrichment followed by host range tests and PCR restriction fragment length polymorphism analysis was used to detect changes in the relative abundance of cyanophage types in June, July, and August 2002. Temporal changes in both the overall composition of the cyanophage community and the relative abundance of specific cyanophage g20 genotypes were observed. In some seawater samples, the g20 gene from over 50% of isolated cyanophages could not be amplified by using the PCR primer pairs specific for cyanomyoviruses, which suggested that cyanophages in other viral families (e.g., *Podoviridae* or *Siphoviridae*) may be important components of the Rhode Island cyanophage community.

Viruses in the marine environment are abundant and diverse. Surface seawater samples typically contain 10^{10} viral particles per liter, and the viral genotypes identified within these samples can be extremely diverse (2, 5, 37). Virus-caused mortality of bacteria and phytoplankton affects levels of dissolved organic matter and inorganic nutrients in seawater and may be a key link in global biogeochemical cycles (5, 32, 37). In addition, viruses have been shown to influence the structure and diversity of marine microbial communities (9).

Cyanobacteria, including the genus *Synechococcus*, are important contributors to primary productivity in marine ecosystems and are found in high concentrations in coastal waters (20, 31). Recent studies have shown that genetically distinct groups of *Synechococcus* spp. can coexist in a single location (4, 17, 27) and that the distribution and abundance of *Synechococcus* spp. vary across locations (4, 19). Cyanophages are viruses that can infect *Synechococcus* spp. and other cyanobacteria. They are ubiquitous in marine environments and are found in great abundance in coastal waters with concentrations of up to 10^8 cyanophages per liter observed in summer months (22, 23, 30). It has been estimated that in coastal waters, 80% of *Synechococcus* cells may encounter infectious cyanophage particles per day (22). Nevertheless, the mortality due to cyanophage infection ranges from only 0.005 to 8% of the *Synechococcus* community per day (7, 15, 22, 30). This low mortality rate suggests that resistance to viral infection is common in *Synechococcus* spp. (7, 22, 30). Indeed, studies indicate that many *Synechococcus* clones isolated from coastal waters are

resistant to cyanophages (22, 30). Even with low mortality rates, cyanophages can still influence the clonal composition of *Synechococcus* communities (30) and could account for some of the cyanobacterial diversity observed in natural communities (25, 27, 37).

Morphological studies show that cyanophages belong to one of three viral families (i.e., *Myoviridae*, *Podoviridae*, or *Siphoviridae*), although the majority of cyanophages isolated from coastal environments have been myoviruses (12, 23, 30). Recently, molecular techniques, including restriction digestion of viral genomes (12, 34), denaturing gradient gel electrophoresis (DGGE) (36), and viral capsid assembly protein (g20) gene sequencing (6, 40), have been used to characterize cyanophage diversity. By the use of these techniques, cyanophage community composition has been examined along a south-north Atlantic Ocean transect (36), in Georgia river estuaries (12, 40), and from stations in the Sargasso Sea (40). These studies have revealed much greater cyanophage diversity than previously reported in studies that were based solely on phenotypic characteristics. For example, using primers specific for the g20 gene sequence of cyanomyoviruses, Zhong et al. (40) identified up to 29 different cyanophage genotypes falling into six phylogenetic clusters in a single water sample. The genetic composition of cyanophage communities appears to be influenced by salinity, light, nutrient availability, depth, and presumably host cyanobacterial populations (12, 22, 36, 40). To date, most studies examining cyanophage diversity have focused on spatial variation. There is little information about how the genetic composition of cyanophage communities may change over time.

In this study we characterized the diversity of cyanophages in Mt. Hope Bay, R.I., over a 3-year period of time. The overall abundance, g20 genotypes, phylogenetic relationships, and host ranges of cyanophages capable of infecting marine *Synechococcus* spp. were determined. We also examined temporal

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and spatial variations in the relative abundances of different cyanophage genotypes in Mt. Hope Bay and Narragansett Bay, R.I.

MATERIALS AND METHODS

Locations and sampling. Surface seawater samples were collected from Mount Hope Bay, R.I., next to the Roger Williams University dock (41°39' N, 71°15' W) every other month from August 1999 through March 2001 and then every month from May 2001 to August 2002. At each sampling date, three 15-ml tubes of seawater were collected several minutes apart to serve as replicate samples. Salinity at this location ranged from 28 to 32 ppt, and the water temperature varied seasonally from 4 to 23°C. To examine spatial variation in the relative abundance of cyanophage genotypes, surface seawater samples were collected from Narragansett Bay at Colt State Park, Bristol, R.I. (44°41' N, 71°17' W), in June and August of 2002 and from the Roger Williams University dock in June, July, and August of 2002.

***Synechococcus* strains.** Four marine isolates of *Synechococcus* spp., WH 7803, WH 8012, WH 8018, and WH 8113, were used in this study. These isolates were obtained from the Woods Hole Collection of Cyanobacteria (Woods Hole Oceanographic Institution, Woods Hole, Mass.) and have been well characterized (16, 19, 28, 31). These four *Synechococcus* sp. strains each belong to a different phylogenetic group based on 16S-23S rDNA internal transcribed spacer sequences (19) and are commonly used to enumerate and isolate cyanophages from natural marine communities (12, 22, 23, 30, 34, 40). *Synechococcus* cells were grown in 250-ml flasks containing SN medium (31) under constant illumination at 20 to 23°C.

Estimation of cyanophage abundance. The abundance of cyanophages capable of infecting *Synechococcus* sp. strains WH 7803, WH 8012, WH 8018, and WH 8113 was estimated using the most-probable-number (MPN) technique. For the MPN assay, serially diluted seawater samples were incubated with *Synechococcus* sp. cells in 24-well microtiter plates as described by Waterbury and Valois (30). An estimate of the concentration of infective cyanophages and error associated with the estimate were determined as described by Hurley and Roscoe (10).

Cyanophage isolation. Seawater samples collected from September 1999 through May 2000 were filtered through a 0.45- μ m-pore-size filter. Starting in July 2000, all water samples were centrifuged for 10 min in a clinical centrifuge at 1,000 \times g rather than being filtered. Enrichment for cyanophages was performed by incubating either 0.1 ml of the filtrate or supernatant with 0.1 ml of *Synechococcus* sp. strain WH 7803, WH 8012, WH 8018, or WH8113 in microtiter plates. After 1 h of incubation, 1 ml of SN medium was added to the wells. Control wells containing host cells, but no seawater dilutions, were included on each plate. Plates were incubated under constant illumination at 20°C, and wells were visually monitored for cell lysis (indicating the presence of virus) for up to 3 weeks. Only wells that completely cleared were considered to be lysed. Lysates were used to isolate individual cyanophages via plaque purification and were also used directly in PCRs.

Each of the four strains of *Synechococcus* spp. was used in the plaque purification of viral isolates. Plaque purification of viral isolates was performed as described by Suttle and Chan (23) with the following modifications. Exponentially growing cyanobacteria were concentrated in 15-ml falcon tubes via centrifugation (1,000 \times g for 10 min). The supernatant was removed, and the cell pellet was resuspended in 2 to 3 ml of SN medium. An aliquot of diluted viral lysate was added to 0.5 ml of concentrated cell suspension. After 30 to 60 min of incubation, 2.5 ml of 0.3% molten (37°C) agar in SN medium was added. The phage-cell-agar mixture was immediately poured onto a 0.6% SN agar plate. Plates were checked daily for the appearance of plaques, which could take up to 3 weeks to form. Viral plaques were picked using sterile toothpicks and transferred to microtiter wells containing 2.5 ml of the appropriate *Synechococcus* strain in SN medium. Viral lysate from these wells was stored at 4°C and was used in the host range tests and in PCR-restriction fragment length polymorphism (RFLP) analysis. A total of 10 to 30 cyanophages were isolated from each water sample. Plates containing concentrated cell suspension but no viral lysate were also included as controls. Viral plaques were never observed on any of these control plates.

Host range. Host ranges of plaque-purified cyanophage isolates were tested by inoculating aliquots of phage lysates to liquid cultures of *Synechococcus* spp. in 24-well microtiter plates as described above for the isolation of cyanophages. Four strains of marine *Synechococcus* spp. were used (WH 7803, WH 8012, WH 8018, and WH 8113). For each virus-host combination, five replicate wells were tested. Controls—containing host cells but no virus—were included on each plate. Plates were incubated as described above and monitored visually for up to 4 weeks for host cell lysis. Cultures that were not lysed after 4 weeks were considered resistant to the virus.

PCR amplification, restriction enzyme digests, and sequencing. Four sets of primers are currently available to amplify overlapping regions of the g20 gene from cyanomyoviruses. Primer set CPS1/CPS2 (6) amplifies a 165-bp region, while CPS1/CPS4 amplifies a ca. 430-bp segment and CPS1/CPS8 amplifies a ca. 592-bp segment (40). The fourth primer set, CPS3/CPS4, appears to be less conserved than the others (40) and was therefore not used in this study. Primer sequences were as follows: CPS1 (5'-GTAGWATTTTCTACATTGAYGTTG G-3'); CPS2 (5'-GGTARCCAGAAATCYTCMAGCAT-3') (6); CPS4 (5'-CAT WTCWTCCCAHTCTTC-3'); and CPS8 (5'-AAATAYTTDCCAACAATGATGG A-3') (40). Amplifications were carried out in either 50- or 25- μ l volumes. The reaction mix contained *Taq* reaction buffer (Invitrogen, Carlsbad, Calif.), 1.5 mM MgCl₂, 100 μ M deoxynucleoside triphosphate (dNTP) (Invitrogen), a 0.2 μ M concentration of each of the primers, 1.25 U of *Taq* DNA polymerase (Invitrogen), and 1 μ l of viral lysate. The viral lysate came from one of three sources: the enrichment, which usually contained more than one viral genotype; plaque-purified viral clones; or wells in the extinction-dilution enrichment. For each set of reactions, a positive control containing a cyanophage known to work with the primers and a negative control sample containing all reagents but lacking viral DNA were included. Control samples containing all reagents and 1 μ l of *Synechococcus* sp. cell culture were also included in PCRs; however, product was never observed in these samples. Reactions were carried out in an Eppendorf Mastercycler gradient (Brinkmann, Westbury, N.Y.) with an initial denaturing step of 2 min at 94°C, followed by 32 cycles of 45 s at 94°C, 45 s at 30°C, 1 min at 72°C, and a final extension step of 4 min at 72°C. All cyanophage isolates were first used in PCRs containing the primer set CPS1/CSP8. If no product was obtained after two tries, the isolates were then used in PCRs containing the primer set CPS1/CPS4. If no product was obtained for primer set CPS1/CPS4, primer set CPS1/CPS2 was then used.

Products of all PCRs were visualized on a 1.5 to 2.5% agarose gel. Cyanophage genotypes were differentiated using a PCR-RFLP technique. PCR products obtained using primer pair CPS1/CPS8 or CPS1/CPS4 were digested with three restriction endonucleases—*Mbo*I, *Rsa*I, and *Mse*I (Invitrogen)—according to the manufacturer's instructions. Only one restriction enzyme was used per reaction. Digested products were separated by gel electrophoresis (2 to 2.5% agarose) and visualized by staining with ethidium bromide. Viral g20 genotypes were differentiated by combining the banding patterns generated by each of the three restriction enzymes.

The g20 gene fragment from cyanophages with different PCR-RFLP profiles was cloned and sequenced. PCR products were cloned using a TOPO TA cloning kit (Invitrogen) following the vendor's instructions. Plasmid DNA was isolated using Qiagen-tip 20 (Qiagen Inc., Valencia, Calif.). For each cyanophage isolate, two clones were usually sequenced with an ABI Prism 377 automated sequencer (PE Applied Biosystems, Foster City, Calif.). M13 forward and reverse primers were used in the sequencing reactions. In a few cases, single-nucleotide differences were observed between clones from the same viral isolate. In these instances, consensus sequences were used.

Phylogenetic analyses. Nucleotide sequences of the g20 gene and the amino acid sequences translated from the nucleotide sequences were aligned for phylogenetic analyses using the ClustalW version 1.7 computer program (26). In addition to the 36 cyanophage g20 sequences from this study, previously described g20 sequences of cyanophage isolates and clones from natural marine communities were also included in the phylogenetic analyses (6, 40). The GenBank accession numbers of these viruses are listed below. The nucleotide and amino acid sequence alignments were unambiguous and contained only one gap of three nucleotides that corresponded to one amino acid. Only the regions flanked by primers CPS1 and CPS4 were used in phylogenetic analyses. Primer sequences were not included in either the nucleotide or amino acid sequence analyses. Using the PAUP software package version 4.0b10 (Phylogenetic Analysis Using Parsimony; Sinauer Associates, Sunderland, Mass.), nucleotide distances were calculated following the Kimura two-parameter model (11) and protein distances were determined based on mean character distances. The PAUP software package was also used to construct phylogenetic gene trees using maximum parsimony and distance methods for the amino acid sequence alignment (143 amino acids).

In the parsimony analysis, 500 replications of the heuristic search option were used with random taxon entry. Tree bisection-reconnection branch swapping was performed, and all characters and character state transformations were unweighted. Distance trees were constructed for amino acid sequences using mean character distances. To compare relative support of the branches, 1,000 bootstrap replications (3) were performed for maximum parsimony and distance analyses.

Relative abundance of cyanophage genotypes. The relative abundance of different cyanophages was determined by diluting seawater to viral extinction before enrichment (i.e., extinction-dilution enrichment) (29). Water samples were

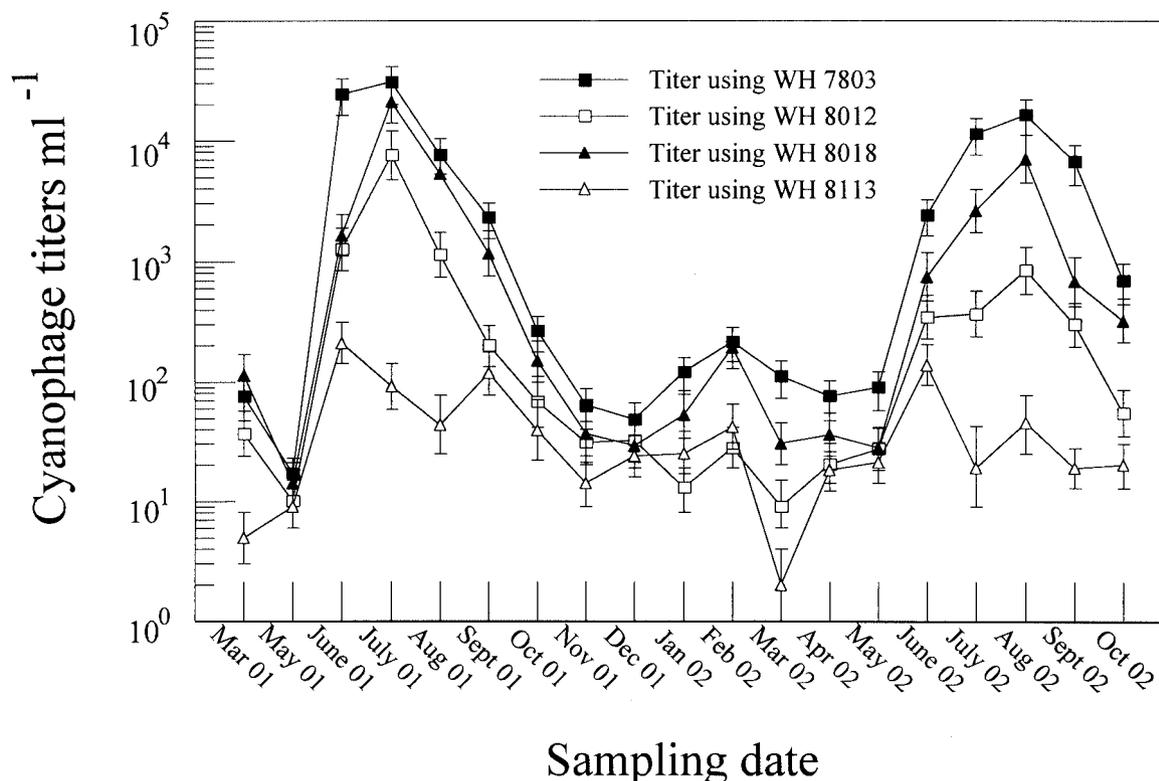


FIG. 1. Cyanophage abundance in seawater samples collected from Mt. Hope Bay, R.I., from March 2001 through October 2002. Estimates of viral titers were determined using the MPN technique with four different strains of marine *Synechococcus* sp. strains WH 7803, WH 8012, WH 8018, and WH 8113.

collected several hours apart from Mt. Hope Bay and Narragansett Bay, R.I., in June, July, and August 2002—except for July, when Narragansett Bay was not sampled. Seawater samples were centrifuged as described above and the supernatant was serially diluted in SN medium. In 24-well microtiter plates, aliquots of the diluted seawater were incubated with *Synechococcus* sp. strain WH 7803. Previous studies have shown that strain WH 7803 is particularly susceptible to a broad range of *Synechococcus* sp. cyanophages (12, 13, 23, 30), and thus this strain was used in the extinction-dilution enrichment. Approximately 80 wells were set up for each dilution (e.g., 10^{-2} , 10^{-3} , or 10^{-4}) for each of the water samples. The dilution in which approximately 50% of the wells lysed was chosen for further analysis. Using the selected dilution, 30 to 40 lysed wells were analyzed per seawater sample. The lysate from each well was analyzed separately in PCR-RFLP assays and in host range tests, thereby allowing the identification of the viral isolate in that well and thus providing an estimate of the relative abundance of the different cyanophage genotypes capable of infecting *Synechococcus* sp. strain WH 7803.

Virus sequences used in the analyses. The GenBank accession numbers of cyanophage g20 sequences used in phylogenetic analyses are as follows: SE1 (AY027985), SE4 (AY027988), SE6 (AY027990), SE9 (AY027995), SE12 (AY027994), SE14 (AY027997), SE31 (AY028008), 27A (AY027974), 31B (AY027975), 32A (AY027976), 44A (AY027977), P12 (AY027980), P17 (AY027981), P77 (AY027982), P79 (AY027983), P81 (AY027984), S-PWM1 (AY027978), S-PM2 (AF016384), S-WHM1 (AF016385), S-BnM1 (AF016386), GS2624 (AY027945), and SS4702 (AY028041).

Nucleotide sequence accession numbers. The g20 gene sequences of cyanophages from Rhode Island have been deposited in GenBank under accession numbers AY259244 to AY259283.

RESULTS

Cyanophage abundance. Estimates of cyanophage concentration ranged from 30,000 phage ml^{-1} in July and August to less than 100 phage ml^{-1} during the winter months (Fig. 1). Within a given seawater sample, the estimated abundance varied with the host strain used in the MPN assay (Fig. 1). The assays

using *Synechococcus* sp. strain WH 7803 consistently provided the highest estimate of cyanophage abundance, which was often twice the estimated abundance obtained when strain WH 8012 or WH 8018 was used as the host. Cyanophage titers estimated using the motile *Synechococcus* sp. strain WH 8113 were often several orders of magnitude lower than that obtained with strain WH 7803 (Fig. 1). Cyanophages capable of infecting each of the four hosts were detected in every water sample.

Characterization of cyanophage isolates. From September 1999 to August 2002, over 350 cyanophage clones were isolated via plaque purification and 210 of these isolates were further characterized. The g20 sequences from 9 of the 210 isolates could not be amplified by any of the primer sets, and therefore these 9 isolates were probably not myoviruses but might be members of one of the other two families of cyanophages—i.e., *Podoviridae* or *Siphoviridae*. Of the remaining 201 cyanophage isolates, 11 isolates could only be amplified using primer set CPS1/CPS2. Due to the short length of the PCR products (165 bp) obtained with this primer set, PCR-RFLP analysis did not yield useful information; however, g20 sequences obtained from two of these isolates (S-RIM37 and S-RIM38) indicated that they were myoviruses (Table 1). PCR product was obtained using primer set CPS1/CPS8 or CPS1/CPS4 for the other 190 cyanophage isolates, and these isolates were identified using PCR-RFLP analysis. In general, digestion of the g20 PCR product resulted in two to four bands per restriction enzyme. All of the cyanophage g20 genotypes contained a restriction site for at least one of the three restriction enzymes used.

Based on PCR-RFLP profiles and host range data, 36 dif-

TABLE 1. Phylogenetic cluster, detection, and host ranges of cyanophage isolates from Rhode Island's coastal waters

Cluster ^a	Cyanophage isolate	Date first detected	No. of times detected ^b	Host range ^c				PCR primers ^d
				7803	8012	8018	8113	
I	S-RIM10	07/2000	1	+	+	+	-	CPS1/CPS8
I	S-RIM11	07/2000	1	+	-	+	+	CPS1/CPS8
I	S-RIM18	08/2001	3	+	+	+	-	CPS1/CPS8
I	S-RIM21	08/2001	4	+	+	+	-	CPS1/CPS8
I	S-RIM23	11/2001	3	+	+	+	-	CPS1/CPS8
I	S-RIM27	05/2002	1	+	+	+	-	CPS1/CPS8
I	S-RIM29	05/2002	2	+	+	+	+	CPS1/CPS8
I	S-RIM32	07/2002	2	+	+	+	-	CPS1/CPS8
I	S-RIM33	07/2002	1	+	-	-	-	CPS1/CPS8
I	S-RIM36	08/2002	1	+	+	+	-	CPS1/CPS8
II	S-RIM5	11/1999	5	-	-	+	-	CPS1/CPS4
II	S-RIM9	05/2000	1	+	-	+	-	CPS1/CPS4
II	S-RIM17	07/2001	4	+	-	-	-	CPS1/CPS4
II	S-RIM20	10/2001	3	+	-	-	-	CPS1/CPS4
II	S-RIM30	06/2002	2	+	-	+	-	CPS1/CPS4
II	S-RIM34	07/2002	1	+	-	+	-	CPS1/CPS4
II	S-RIM35	08/2002	1	+	-	+	-	CPS1/CPS4
III	S-RIM1	09/1999	4	+	+	+	-	CPS1/CPS8
III	S-RIM2	09/1999	9	+	+	+	-	CPS1/CPS8
III	S-RIM3	09/1999	8	+	-	+	-	CPS1/CPS4
III	S-RIM4	09/1999	4	+	+	-	-	CPS1/CPS8
III	S-RIM7	03/2000	4	+	+	+	-	CPS1/CPS8
III	S-RIM8	03/2000	13	+	-	+	-	CPS1/CPS4
III	S-RIM12	09/2000	18	+	+	+	-	CPS1/CPS8
III	S-RIM13	11/2000	1	+	+	+	-	CPS1/CPS8
III	S-RIM14	03/2001	8	+	+	+	-	CPS1/CPS8
III	S-RIM15	03/2001	1	+	+	+	-	CPS1/CPS8
III	S-RIM16	05/2001	2	+	-	+	-	CPS1/CPS8
III	S-RIM19	08/2001	2	+	-	+	-	CPS1/CPS8
III	S-RIM22	09/2001	2	+	-	+	-	CPS1/CPS8
III	S-RIM24	12/2001	7	+	-	+	-	CPS1/CPS4
III	S-RIM25	02/2002	1	-	-	+	-	CPS1/CPS8
III	S-RIM26	02/2002	1	-	-	+	-	CPS1/CPS8
III	S-RIM28	06/2002	1	+	-	+	-	CPS1/CPS8
III	S-RIM31	06/2002	3	+	+	+	-	CPS1/CPS4
UG	S-RIM6	11/1999	5	+	+	+	-	CPS1/CPS8
NA	S-RIM37	11/1999	NA	+	-	-	-	CPS1/CPS2
NA	S-RIM38	01/2000	NA	+	-	-	-	CPS1/CPS2
NA	S-RIM39	07/2002	NA	+	+	+	-	CPS1/CPS2
NA	S-RIM40	08/2002	NA	+	-	+	-	CPS1/CPS2

^a Cluster refers to phylogenetic group in Fig. 2. UG, ungrouped; NA, not available.

^b Out of a possible 26 sampling dates.

^c *Synechococcus* sp. strains WH 7803, WH 8012, WH 8018, and WH 8113 were used. +, infection occurs; -, no infection.

^d Primer set used to amplify the viral g20 gene for sequence analysis.

ferent cyanophage g20 genotypes were identified (Table 1). At least one isolate representing each RFLP banding pattern was sequenced. To double check genotyping, viral isolates with identical PCR-RFLP profiles that were isolated months or even years apart were sequenced. Seven pairs of isolates were tested in this manner. In all cases but one, the sequences were identical or differed by no more than two nucleotides from each other, thus suggesting that PCR-RFLP analysis can be used to distinguish cyanophage g20 genotypes. In the one exception, the cyanophage genotypes were distinguishable based on host range.

The g20 gene from 11 of the 36 Rhode Island cyanophage genotypes could not be amplified using primer set CPS1/CPS8 but were able to be amplified using the primer set CPS1/CPS4 (Table 1). In some cases, the converse was also true: some viral isolates could be amplified with primer set CPS1/CPS8 but not with CPS1/CPS4. Our results indicate that primer set CPS1/

CPS2 was able to amplify a broader range of cyanophages than either the CPS1/CPS8 or CPS1/CPS4 primer set.

The majority of the isolated cyanophages could infect more than one *Synechococcus* sp. strain (Table 1). Host range tests of the 36 cyanophage genotypes revealed that 92% of the genotypes could infect *Synechococcus* strain WH 7803, while 88 and 50% could infect WH 8018 and WH 8012, respectively (Table 1). Only 2 of the 36 genotypes were able to infect WH 8113. Although cyanophages infecting *Synechococcus* sp. strain WH 8113 were detected in the MPN assays, these viruses did not easily form plaques when plated on WH 8113 cells, and those viruses that were isolated via plaque purification typically could not be amplified with the cyanomyovirus g20 primers.

Phylogenetic analyses of g20 sequences. Phylogenetic analyses of the viral g20 amino acid sequences produced maximum-parsimony and distance trees with similar overall topologies. The main differences between these trees were the branching

orders of terminal taxa within a clade. Cyanophage sequences from Zhong et al. (40) included in the analyses were used to identify the phylogenetic clusters defined in that study. Clusters I, II, and III were present in all the distance and maximum-parsimony trees, although cluster II had low bootstrap support (<50%) in the parsimony analysis. Thirty-five of the 36 Rhode Island cyanophages fell into cluster I, II, or III, while one cyanophage isolate (S-RIM6) was a sister taxon to clusters I and II (Fig. 2). It should be noted that in the classification of cyanophages per Zhong et al., cyanophage isolate P17 was not placed into any of the clusters (40). We have expanded cluster III to include P17 along with four related cyanophage isolates identified in Rhode Island's coastal waters (Fig. 2).

Nucleotide sequence similarities among the Rhode Island cyanophage isolates ranged from 47.3 to 98.6%, while the amino acid sequence similarities ranged from 70.4 to 100%. Five pairs of Rhode Island cyanophages with g20 nucleotide sequence similarities ranging from 90.4 to 98.6% and different PCR-RFLP profiles had identical amino acid sequences (Fig. 2). Four Rhode Island cyanophage isolates had g20 amino acid sequences that were identical to cyanophage sequences available in GenBank. The amino acid sequences of Rhode Island cyanophage isolates S-RIM18 and S-RIM23 are identical to the amino acid sequences of six cyanophage clones (i.e., SE4, SE6, SE9, SE12, SE14, and SE31) from a natural virus concentrate of surface seawater collected from the pier of the Skidaway Institute of Oceanography in Savannah, Ga. (40). The amino acid sequence of Rhode Island cyanophage S-RIM1 is identical to the cyanophage isolate S-PWM1, which was originally isolated from the Gulf of Mexico (23, 40). Finally, cyanophage isolate S-RIM2 has an identical g20 nucleotide—and thus amino acid—sequence to cyanophage isolate S-WHM1, which was isolated from seawater collected from Woods Hole Harbor, Woods Hole, Mass. (6, 34) (Fig. 2).

Temporal variation in the genetic composition of the cyanophage community. In Rhode Island's coastal waters, 36 different cyanophage g20 genotypes were identified over a 3-year period (Table 1). Nevertheless, on average only five g20 genotypes were detected in each seawater sample. For each sampling date, approximately 10 plaque-purified cyanophage isolates were analyzed using PCR-RFLP analysis. Beginning in March 2001, PCR-RFLP analysis was also performed using viral lysates directly from enrichment wells, and one to three viral genotypes were typically identified in each lysate. Viral g20 genotypes of plaque-purified viruses were often different from the g20 genotypes detected using PCR-RFLP analysis of enrichment lysates, even though plaque purification was performed using dilutions of enrichment lysates. This may be due to variability in PCR priming efficiency for different cyanophages (6) and/or to the ability of particular viral genotypes to form plaques on soft agar plates. For example, cyanophage genotype S-RIM12 was readily detected via PCR-RFLP analysis of viral lysates; however, this virus took up to 3 weeks to form tiny plaques on soft agar plates. Other viral genotypes—for example, S-RIM7—had a large plaque morphology that led to confluent plate lysis after approximately 2 weeks. If S-RIM12 and S-RIM7 were present in the same water sample, plaques of S-RIM7 would likely lyse the plate before S-RIM12 had a chance to form plaques.

The smallest number of different cyanophage g20 genotypes

was detected in the seawater samples collected in January. Only one cyanophage genotype was detected in January 2000 and 2001, and two genotypes were detected in January 2002. Between five to seven different cyanophage genotypes were detected in seawater samples collected in July, August, or September. Typically, several new cyanophage g20 genotypes that had not previously been observed were identified in each seawater sample. After the first year of sampling, 11 cyanophage genotypes had been identified. During the second year of sampling, an additional nine genotypes were detected, and in the final year 16 new cyanophage genotypes were characterized (Table 1). Although new viral genotypes were continually being identified, some viral genotypes were repeatedly observed (Table 1). For example, cyanophage genotype S-RIM12 was first identified in September 2000 and was then detected in all but two subsequent water samples. Overall, 23 of the 36 viral genotypes were detected in seawater samples from two or more sampling dates (Table 1). Seasonal patterns in the appearance and disappearance of particular genotypes were not detected, perhaps due to insufficient sampling. There was also no correspondence between season and the presence of genotypes in different phylogenetic groupings. Cyanophage genotypes representing each of the three main phylogenetic groups (i.e., clusters I, II, and III) (Fig. 2) were frequently found in the same seawater sample.

Relative abundance of cyanophage types. To gain an estimate of the proportion of cyanophages that were not detected by PCR-RFLP analysis, extinction-dilution enrichment followed by PCR-RFLP analysis and host range tests were used to estimate the relative abundance of cyanophages in seawater samples collected in June, July, and August of 2002. By using this method, we detected 10 to 16 different cyanophages in each of the samples. However, the g20 genes from only four to nine of the viral types could be amplified using primer set CPS1/CPS8 or CSP1/CPS4 and then subsequently genotyped (Fig. 3). Between 15 and 38% of the total number of cyanophages in a sample could only be amplified with primer set CPS1/CPS2. The g20 gene fragment (165 bp) from two of these viruses was sequenced (S-RIM39 and S-RIM40; Table 1). Based on sequence similarity, they were identified as myoviruses. Unfortunately, the fragment produced by CPS1/CPS2 is too short for use in phylogenetic analyses (40); thus, the rest of these viruses were distinguished based on host range and placed into one of five groups—designated RIMA through RIME (Fig. 3). Depending on the sample, 2 to 52% of the total number of isolated viruses was unable to be amplified using any of the three g20 cyanomyovirus primer sets. These viruses were also placed into one of five groups, labeled RIUA through RIUE, based on host range (Fig. 3).

The seawater samples collected in June 2002 from Mt. Hope Bay and Narragansett Bay, R.I., contained a similar set of cyanophage g20 genotypes, as did the two August seawater samples from both these locations (Fig. 3). However, there were striking differences in the types and relative abundance of cyanophages detected in June and those detected in August. For example, cyanophage genotypes S-RIM14, S-RIM12, S-RIM29, and S-RIM28 were present in the June seawater samples from Mt. Hope Bay and Narragansett Bay and, when combined, represented more than 47% of each sample; however, none of these cyanophage genotypes was detected in

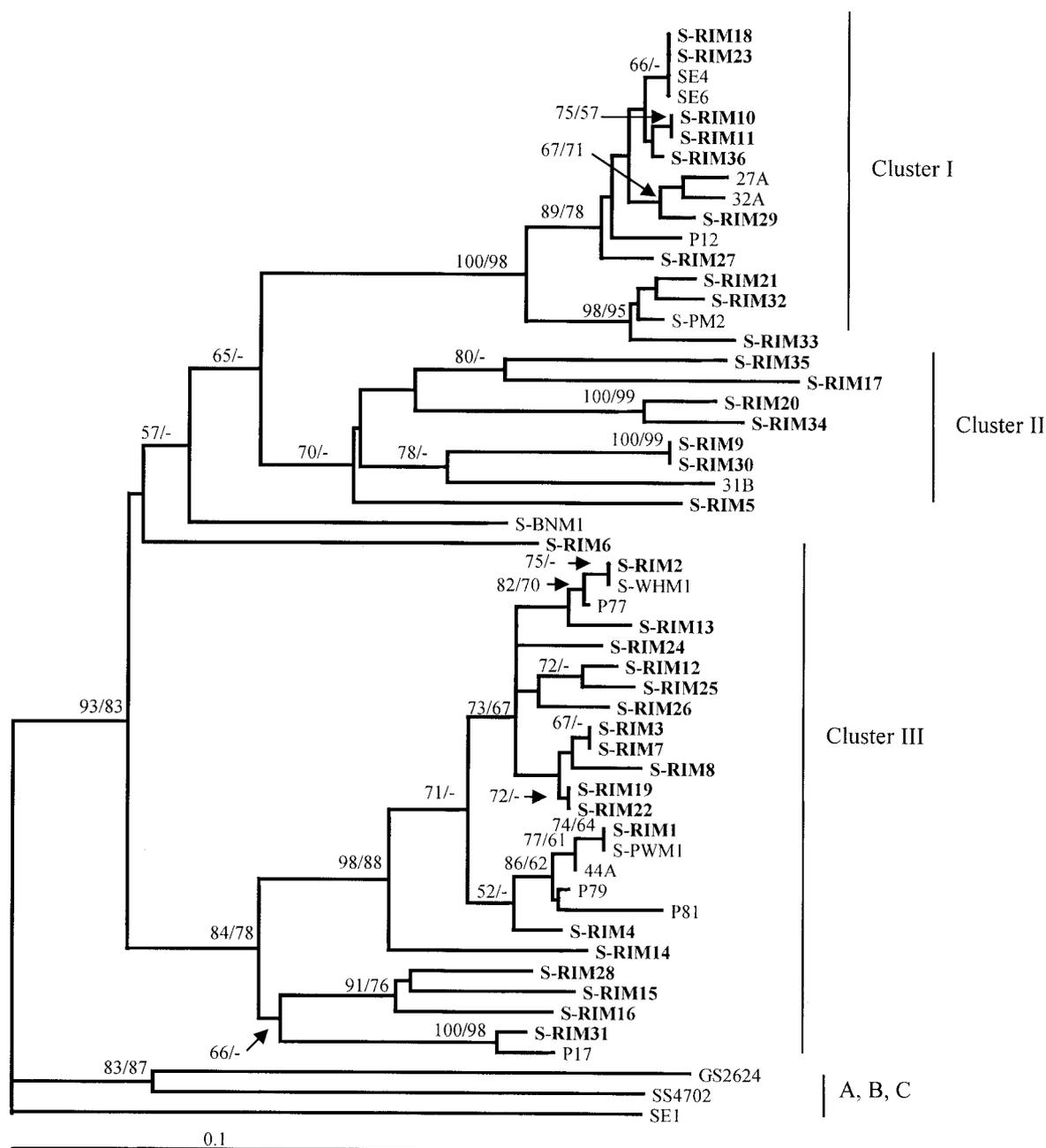


FIG. 2. Phylogenetic relationships of cyanophage isolates from Rhode Island and selected other cyanophage sequences. The tree is based on the 143-amino-acid sequence alignment from the viral g20 gene and was constructed using the neighbor-joining method. Cyanophage isolates from Rhode Island's coastal waters are in boldface. Clusters were assigned based on the classification scheme of Zhong et al. (40). Bootstrap values (neighbor-joining and parsimony) are indicated at the nodes with more than 50% bootstrap support. Tree is rooted with cyanophage clone SE1 (GenBank AY027985).

either of the August seawater samples (Fig. 3). Also interesting was the high relative abundance (36%) of cyanophage genotype S-RIM34 in the July Mt. Hope Bay sample, since this genotype was not detected in either of the June or August samples (Fig. 3). In fact, this was the only time out of the 26 sampling dates that S-RIM34 was detected (Table 1). Our results indicate that in Rhode Island's coastal waters, temporal variation in the genetic composition of the cyanophage community may be greater than spatial variation, as was observed

in an earlier study (P. Brannock and M. Marston, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. 191, 2002).

DISCUSSION

The cyanophage community in Rhode Island's coastal waters is genetically diverse and dynamic. Cyanophages could easily be detected and isolated from seawater throughout the year. The cyanophage titers observed in Mt. Hope Bay, ranging from over 10^4 phage ml of seawater⁻¹ during the summer

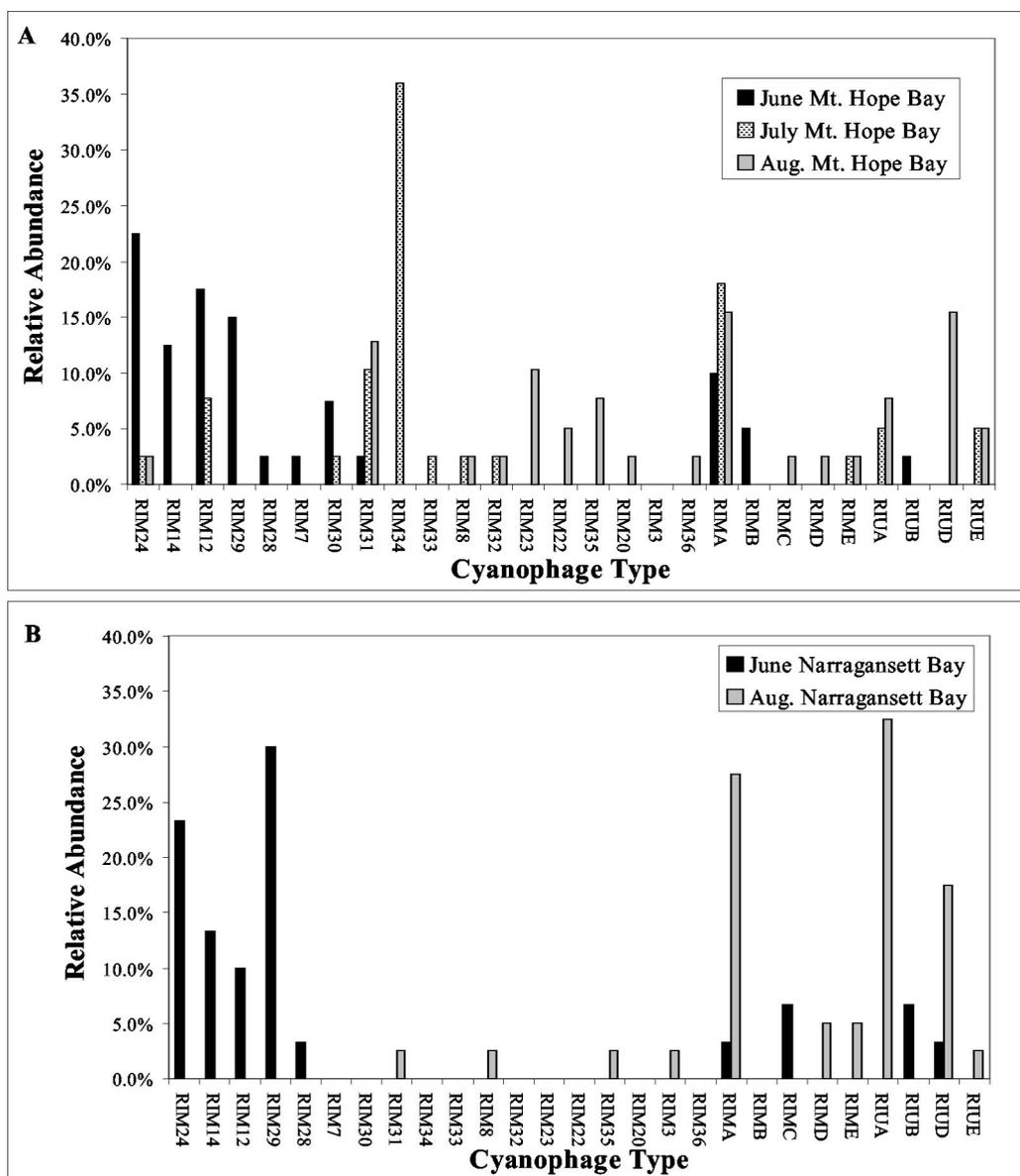


FIG. 3. Relative abundance of cyanophage g20 genotypes in Mt. Hope Bay (A) and Narragansett Bay (B) seawater samples. Samples were collected in June, July, and August of 2002. RIMA through RIME are myoviruses that could not be identified by PCR-RFLP analysis and thus were distinguished based on host range. RIUA through RIUE are cyanophages whose identity is not known, but they were also distinguished based on host range.

months to less than 10^2 phage ml^{-1} in winter months, were similar to those reported from a variety of other coastal locations (7, 12, 22, 30). The seasonal variability in cyanophage numbers has been correlated with the annual cycles of *Synechococcus* spp. abundance, which is influenced by changes in water temperature (31). As observed in other studies (12, 22, 23, 30), estimates of cyanophage abundance varied depending on the host strain used in the MPN assay due to differences in viral host ranges.

While seasonal changes in cyanophage abundance have been well documented, little is known about if or how the genetic composition of a cyanophage community changes over time. In this study, the genetic composition of the cyanophage community in Mt. Hope Bay was monitored over 3 years. Previous

studies of the genetic diversity of cyanophages have examined viral isolates from different locations collected at a single time point (6, 12, 34) or have used DGGE or clone libraries to compare samples collected at a number of geographically distinct locations (36, 40). While the use of DGGE and clone libraries allows for estimates of viral genetic diversity without the need to culture host organisms, these methods will only detect viruses that can be successfully amplified using the chosen PCR primer set. Therefore, it is often unclear how many additional viruses may be present yet not detected. In addition, the host ranges and relative abundances of the viruses are usually not known. By first isolating cyanophages and then characterizing them, we were able to identify cyanophages that would have been missed in clone libraries that had been con-

structured using a single primer set. One drawback of this method is that we were only able to characterize cyanophages capable of infecting *Synechococcus* sp. strain WH 7803, WH 8012, WH 8018, or WH 8113. Nevertheless, the methods used in this study, in combination with DGGE or clone library data, should allow for a more complete picture of cyanophage community diversity.

Sequence analyses of the viral g20 gene have been used to analyze the diversity of natural assemblages of marine cyanomyoviruses (6, 13, 36, 40). This gene encodes a virion head portal protein that is involved in capsid assembly (8) and is part of a conserved region found in most cyanomyoviruses (6, 8, 13). Although conserved sequences have allowed PCR primers to be designed, significant sequence diversity has been documented among g20 genes from marine cyanophages (6, 36, 40). In Rhode Island coastal waters, 36 distinct cyanomyoviral g20 nucleotide sequences were identified by using primer pairs CPS1/CPS4 and CPS1/CPS8. These viral g20 sequences are genetically diverse and were able to be distinguished based on PCR-RFLP profiles. Phylogenetic analyses of the Rhode Island cyanophage g20 sequences placed them into three main clades that were previously designated as clusters I, II, and III by Zhong et al. (40). Most known cyanophage isolates of marine *Synechococcus* spp. and a number of viral clones from both estuarine and open-ocean environments also belong to cluster I, II, or III (6, 12, 36, 40), although g20 sequences belonging to six additional clades have also been identified in clone libraries of natural marine communities (40). It had been suggested that the hosts of cyanophages in cluster II may be adapted to oligotrophic environments (40); however, seven cyanophage g20 genotypes belonging to cluster II were identified in Mt. Hope Bay—a eutrophic environment. In fact, cyanophages belonging to each of the three phylogenetic clusters were frequently detected in the same seawater sample.

The diversity of cyanophages in a single water sample was much lower than the total cyanophage diversity observed over the 3-year study period. A total of 36 different cyanomyovirus g20 genotypes were identified, but only 1 to 9 different g20 genotypes were detected at each sampling date. In general, seawater collected during the summer months had a greater number of different viral g20 genotypes than seawater samples collected during the winter months. When analyzing viral clones made from concentrated water samples, Zhong et al. (40) identified between 1 and 16 cyanophage g20 genotypes belonging to cluster I, II, or III in a sample. It is interesting that these different methods used to analyze water samples from very different locations give approximately the same number of cluster I, II, and III cyanophages per sample.

Cyanophage decay rates in natural environments are on the scale of hours to days (7, 22); therefore, lytic viruses should only persist in the environment if the average time required to contact and infect a host is less than the viral decay rate. Our results indicate that two cyanophage g20 genotypes were being produced in high enough abundance (on the order of several thousand infectious viral particles per liter of seawater) that they could be detected without concentration from most water samples for over 2 years. There are several possibilities that might serve to explain the high abundance of these cyanophage genotypes over time. One possibility is that these cyanophages coexist stably with their hosts. Even if most host genotypes are

resistant to infection (23, 30), cyanophages could persist by scavenging on a few susceptible cells (30, 37). Another possibility is that coevolution is occurring. As hosts evolve resistance to cyanophages, host range mutants of the virus may arise that are able to overcome the resistance (1, 24). A third possibility is that cyanophages may switch hosts as the relative abundance of *Synechococcus* spp. changes seasonally or as some *Synechococcus* sp. hosts acquire resistance. Thirty of the 36 cyanophage g20 genotypes isolated from Mt. Hope Bay were able to infect more than one strain of *Synechococcus* spp. and it has been reported that cyanophages infecting *Synechococcus* spp. in marine cluster A typically have broad host ranges—often capable of infecting over 10 different host strains (12, 23, 30). There is also genetic evidence to suggest that several different *Synechococcus* spp. can coexist in the same water sample (4, 17, 27). Therefore, it seems likely that a cyanophage may rely on several different host *Synechococcus* spp.

Finally, persistence of a cyanophage could be due to lysogenic or pseudolysogenic interactions between the virus and its hosts. Several studies suggest that lysogeny may be an important type of phage-host interaction in marine environments (18, 33, 35), and lysogenic interactions between marine *Synechococcus* spp. and cyanophages have been documented (14, 15, 21). In this case, continual viral production could be a result of the induction of prophage from lysogenic cyanobacteria. These possibilities are not mutually exclusive, and it is possible that several different mechanisms enable a cyanophage to persist in high abundance throughout the year. It is unknown if the 13 cyanophage g20 genotypes that were detected only once were always present—but in very low abundance and thus were only detected when their relative abundance increased—or if new viral genotypes were continually being introduced into the system.

It has been suggested that most marine *Synechococcus* cyanophages are myoviruses (12, 13, 23), and to date, most genetic studies of marine cyanophages have focused on myoviruses (36, 40). However, in our extinction-dilution assays, PCR product could not be amplified from 2 to 52% of the isolated viruses using any of the three myoviral g20 primer sets. These results suggest that other viral types (perhaps belonging to the *Podoviridae* or *Siphoviridae* viral family) may be an important component of the Rhode Island cyanophage community. Moreover, there appears to be temporal variation in the relative abundance of these other cyanophage types.

Temporal variation was observed in both the relative abundance of specific g20 genotypes and the overall composition of the cyanophage communities in Mt. Hope Bay and Narragansett Bay over a 3-month period of time. The cause of the observed changes in the relative abundance of specific cyanophage genotypes is not known. Nevertheless, these data are consistent with models that predict that the overall structure of viral communities should be dynamic and that peaks in the abundance of a dominant virus may be short lived (25, 39). Using pulsed-field gel electrophoresis to monitor changes in the relative abundance of different-sized viral genomes over time, Wommack et al. detected temporal and spatial variations in Chesapeake Bay viral communities (38) and observed episodic blooms in the abundance of specific viral types (39). They suggested that these viral blooms begin when host cell density reaches a critical abundance and then decline when host cell

density is reduced due to viral lysis (39). Our results indicate that specific cyanophage genotypes, all infecting the same host species, can also undergo episodic blooms. The temporal changes that we observed in cyanophage genotypes suggest a dynamic interaction between cyanophage communities and *Synechococcus* communities that likely occurs over a time scale of weeks. The results from this study provide a starting point to begin to correlate changes in cyanophage community composition with possible changes in the diversity and composition of the local host *Synechococcus* community.

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