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A temporal and spatial investigation of cyanophage abundance in the Gulf of Aqaba, Red Sea

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The aim of this study was to determine the abundance of cyanophages over an annual cycle in the Red Sea from the period April 1999 to December 1999 at a range of depths. Cyanophage numbers from 71 water samples were determined by the use of plaque assays using four different *Synechococcus* strains. The results indicate that cyanophage are found throughout the water column from surface waters to depths of 150 m, with a discrete maximum in the number of cyanophages in the summer months of July, August and September at a depth of 30 m. Eighty-seven cyanophages were isolated and characterized in terms of host range, genome size and possession of a myoviral portal vertex gene. Cyanophages were found to infect multiple strains of *Synechococcus* from different phylogenetic clades. The genome sizes of cyanophages were also found to be bigger than previously estimated.

INTRODUCTION

Cyanophages are bacteriophages capable of infecting cyanobacteria. They are ubiquitous in the marine environment and have been isolated from a number of diverse locations that include the English Channel, Sargasso Sea and the Gulf of Mexico (Suttle & Chan, 1993; Waterbury & Valois, 1993; Wilson et al., 1993; Suttle & Chan, 1994; Sullivan et al., 2003). Cyanophages infecting marine *Synechococcus* strains have been the object of study for over ten years and more recently cyanophages infecting *Prochlorococcus* have also been isolated and characterized (Sullivan et al., 2003). The influence that cyanophages can have on marine ecosystems may be broader than previously thought with the recent discovery that cyanophages contain genes that encode proteins essential for photosynthesis, suggesting they may influence the photosynthetic ability of infected hosts (Mann et al., 2003).

Previous studies have shown that cyanophage titres change on a seasonal basis (Waterbury & Valois, 1993). Whilst others have found that cyanophage diversity changes on a spatial scale (Frederickson et al., 2003). This study aimed to investigate the abundance of cyanophages both temporally and spatially in the Gulf of Aqaba in the Red Sea. The Gulf of Aqaba is separated from the Straits of Tiran by a shallow sill at a depth of 250 m, which restricts the flow of Red Sea water into the Gulf. This causes a long residence time for water in the Gulf, with the surface water resident for one year and deeper waters between 3 and 8 years (Reiss & Hottinger, 1984), and combined with a lack of drainage into the straits results in a body of water that although coastal in geographical terms is representative of open ocean water in terms of its nutrient status (Klinker et al., 1976; Wolfvecht et al., 1992). There is a distinct seasonal pattern to the circulation of water in the Gulf, which is characterized by stratification of the water column in the summer months, which disappears and by winter the water column is mixed as nutrient-rich water has risen to the surface (Klinker et al., 1976; Wolfvecht et al., 1992). The *Synechococcus* and *Prochlorococcus* community within the Gulf of Aqaba has previously been studied in detail (Fuller et al., 2003, 2005). The diversity of the *Synechococcus* in the Red Sea has previously been found to co-vary with cyanophage diversity indicating that cyanophage diversity in the Red Sea can have a controlling influence on *Synechococcus* diversity (Mühling et al., 2005).

MATERIALS AND METHODS

Host strains and media

The host *Synechococcus* strains used in this study were maintained in artificial seawater (ASW) medium (Wyman et al., 1985) in 100 ml batch cultures in 250 ml conical flasks under constant illumination (5–36 μmoles $m^{-2} s^{-1}$) at 25°C. Larger volumes were grown in litre and 2-l vessels to which 0.5 g l^{-1} of $NaHCO_3$ was added. Cultures were aerated and stirred constantly. The *Synechococcus* strains used in this study were WH7803, WH8103, RS9906 and RS9911. These four strains were representatives of four different phylogenetic clades as determined by 16S rRNA sequence analysis (Fuller et al., 2003). *Synechococcus* sp. WH7803 and WH8013 are both well characterized strains and have been in culture for over 20 years. *Synechococcus* sp. RS9906 and RS9911 were both isolated by N. Fuller from the Gulf of Aqaba at the same time as water samples were collected during the period February 1999 to January 2000 (Fuller et al., 2003). *Prochlorococcus* sp. SS120 was cultured in the enriched seawater medium PCRS-11 (Partensky et al., 1999) in 100 ml volumes in 175 ml volume Nunclon plastic flasks (Nalgene Nunc International, Rochester, NY) or in 5 ml

volumes in plastic bijoux at 21°C under constant illumination (5–36 μmoles m^{-2} s^{-1}) without stirring.

Location and sampling

A total of 71 water samples was kindly collected over a nine-month period by N. Fuller from the Gulf of Aqaba at Station A (29°28′N 34°55′E) between April 1999 and December 1999. Only single water samples were collected for each sampling point which varied in depth from surface waters down to 150 m. Water was first passed through a Supor-450 filter (0.45-μm pore size, Gelman Sciences, Inc., Ann Arbor, Michigan) under a vacuum of 10 mm Hg. Water samples were then passed through a filter of pore size 0.20 μm (Sartorius, Epsom, UK) by the use of a syringe and stored in the dark at 4°C until ready for use. Samples were collected at depths of 10, 30, 50, 80, 100 and 150 m on the 19 & 27 April, 4 June, 18 July, 23 August, 7 September, 4 & 18 October, 22 November and 27 December 1999. Additional samples were collected from 60 m on the 19 & 27 April and 14 June 1999; 125 m on 19 April, 14 June and 18 October 1999. On 11 May 1999 from depths of 0, 50, 80, 100 and 150 m.

Estimation of cyanophage abundance

Cyanophage abundance was elucidated from these water samples by the use of plaque assays (Wilson et al., 1993). A minimum of three replicates were used in the enumeration of cyanophage numbers for all 71 water samples. All four *Synechococcus* strains were used to enumerate cyanophage numbers. The number, size and morphology of the plaques obtained were recorded.

Isolation of cyanophages

Cyanophages were isolated by the removal of a plaque with a Pasteur pipette and resuspension in 1 ml of ASW. This phage preparation was then used in a further round of single plaques isolation, using serial dilutions if necessary. This process was repeated a total of three times to ensure that clonal isolates were obtained. Isolates were named following the previously suggested convention of Suttle (Suttle, 2000), with the letters RS used to designate the location as the Red Sea.

Determination of cyanophage host range

Host ranges were determined for the clonal stocks of cyanophage isolated from the Red Sea. Cyanophages were tested for infectivity against all four strains of *Synechococcus* and *Prochlorococcus* sp. SS120. Infection of *Synechococcus* sp. WH7803, WH8103 and RS9906 was tested by means of plaque assays; infection of *Synechococcus* sp. RS9911 was tested by means of well assays (Suttle & Chan, 1993). Well assays were used for RS9911 as it is easier to culture this strain in liquid than on solid agar plates. For testing infection of *Prochlorococcus*, the phage lysate was added to triplicate samples of 5 ml of exponentially growing culture. Phages S-WHM1 and S-PM2, previously isolated from Woods Hole and Plymouth respectively (Wilson et al., 1993), were used as negative controls as they had been shown not to infect *Prochlorococcus* sp. SS120 (Sullivan et al., 2003).

DNA extraction and PCR amplification

DNA was extracted from cyanophage preparations obtained by the propagation of cyanophages to produce confluent lysis of lawns of *Synechococcus*. The top layer of 0.4% agar from five or more plates was pooled and resuspended in 2 ml of ASW, this was then centrifuged at 13,000 × *g* to pellet the agar. DNA was then extracted from the supernatant (Wilson et al., 1993); this briefly comprised a phenol extraction, followed by a phenol/chloroform (1 : 1) and an iso-amyl alcohol/chloroform (1 : 24) extraction. DNA was then precipitated by the addition of ammonium acetate and propan-2-ol, before being washed with 70% ethanol and resuspended in 50 μl H_2O.

Polymerase chain reaction (PCR) using the extracted DNA as a template was employed to screen phage isolates in order to aid in classification of the phages. The cyanomyovirus-specific g20 primers CPS-1 and CPS-2 were employed in a PCR using previously described conditions (Fuller et al., 1998) and the products were analysed on a 1% agarose gel. The presence of a PCR product of the correct size indicated that the phage was a member of the family Myoviridae. Phages that could not be identified as myoviruses by the use of PCR were examined by transmission electron microscopy (TEM) in order to characterize their morphology.

Transmission electron microscopy

Samples from cyanophage stocks were examined in a Joel Jem-100s TEM. Grids were first coated in butvar film (Sigma); a microscope slide was immersed in a solution of 1.5% (w/v) butvar/chloroform for 30 s, then removed and allowed to dry in the vapour of the chloroform. Several 200 mesh copper grids (Agar Scientific, Essex, UK) were placed shiny face down onto the butvar film and dried in an oven at 50°C for one hour. Fifty μl of phage lysate was placed onto a butvar coated copper grid and left to absorb for 20 min. The liquid was then carefully removed with blotting paper and 20 μl of 2% (w/v) uranyl acetate was added to the grid and immediately removed. A further 20 μl of uranyl acetate 2% (w/v) was added and left for 20 s before being removed. Grids were examined at an accelerating voltage of 80 kV and images were obtained at magnifications of × 40,000–100,000. The addition of 0.012 μm diameter polystyrene beads (Agar Scientific, Essex, UK) served as a control in the measurement of phage particles.

Determination of genome size

The genome size of cyanophage isolates was determined by the use of pulsed-field gel electrophoresis (PFGE). High titre suspensions of cyanophage isolates were produced by the removal of the top layer of 0.4% (w/v) agar from a plaque assay where confluent lysis had occurred. The top layer of 0.4% (w/v) agar was resuspended in 1 ml of ASW, left overnight at 4°C to allow phage diffusion, and then centrifuged at 13,000 × *g* for 5 min to pellet the agar. The supernatant was removed and used to make agarose/phage plugs by using 50 μl of phage lysate added to 50 μl of molten 2% low melting point agarose (Sigma) allowing it to set in plug moulds (Bio-Rad). Once set, the plugs were

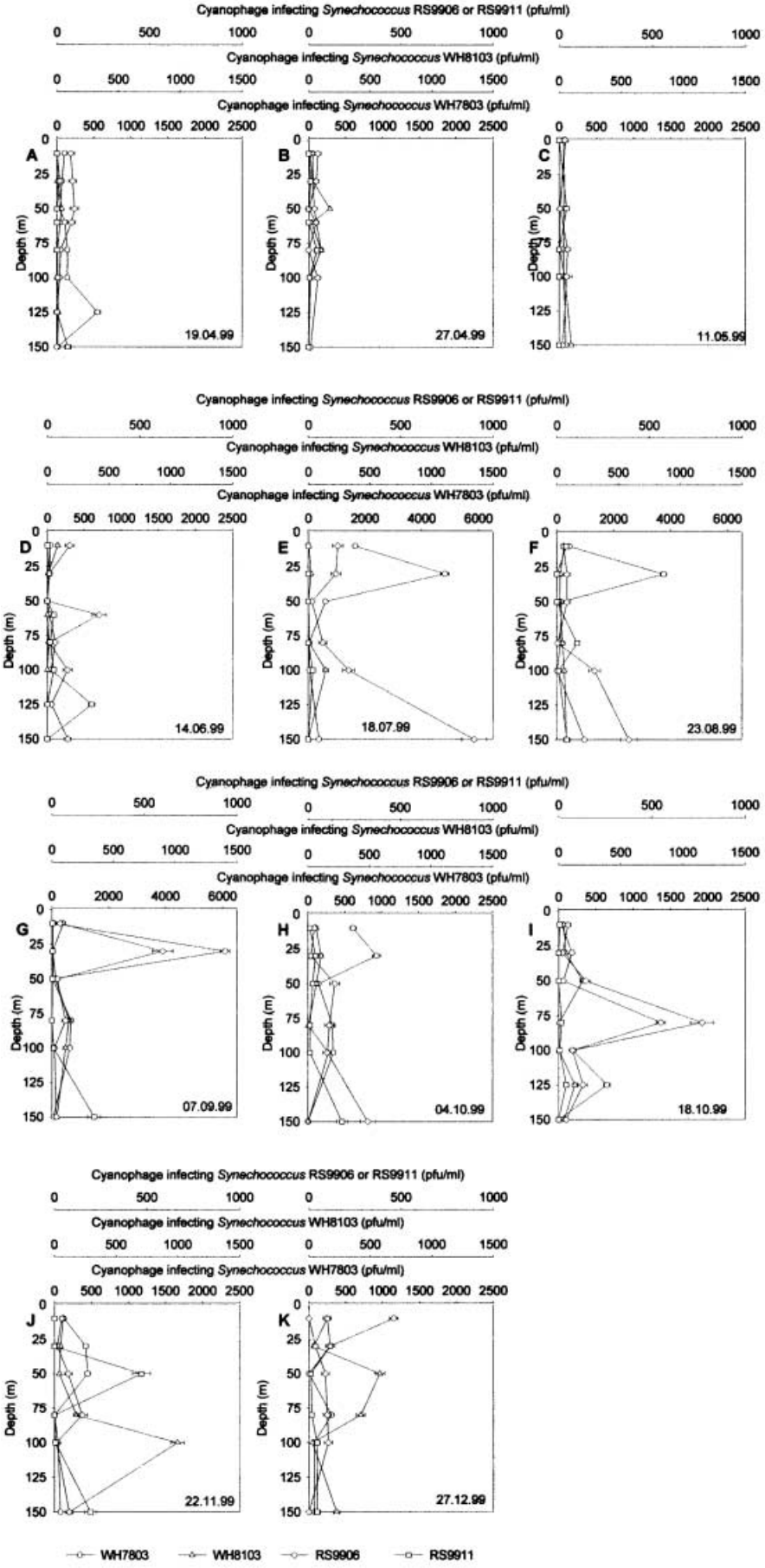

Figure 1. Depth profiles of the distribution of cyanophages (pfu/ml) in the Gulf of Aqaba from 19 April 1999 to 12 December 1999 from water samples collected from 0 to 150 m in depth. Cyanophage abundance was determined by the use of plaque assays using *Synechococcus* strains WH7803 (○), WH8103 (△), RS9906 (◇) and RS9911 (□) as hosts.

Table 1. *Characterization of cyanophages from the Red Sea. Cyanophages were named using the nomenclature suggested by Suttle (2000); S (*Synechococcus*), RS (Red Sea), M (Myovirus) and 3 (isolate number). The date and depth the water sample used for isolation was collected from. PCR using cyanomyovirus specific primers CPS-1 and CPS-2 was used to identify the phages as cyanomyoviruses. Genome size was determined using PFGE. The host range of the cyanophages against* Synechococcus *sp. WH7803, WH8103, RS9906 was determined by plaque assays and well assays against RS9911 and* Prochlorococcus *SS120.*

Phage	Date of isolation	Depth	Genome size	Gp20 product	Host range WH7803	WH8103	RS9906	RS9911	SS120
S-RSM3	7.9	150	154 ±6	√	●	[●]	○	○	○
S-RSM4	27.4	50	154 ±6	√	●	[●]	○	○	○
S-RSM5	27.12	150	170 ±5	√	○	[●]	○	○	○
S-RSM6	19.4	50	155 ±10	√	●	[●]	○	○	○
S-RSM7	27.4	10		√	○	[●]	○	○	○
S-RSM8	11.5	0	161 ±8	√	●	●	●	○	○
S-RSM9	19.4	0	155 ±4	√	●	[●]	○	○	○
S-RSM10	19.4	125		√	[●]	○	○	○	○
S-RSM11	27.4	10	153 ±3	√	●	[●]	○	○	●
S-RSM12	27.12	30		√	[●]	○	○	○	○
S-RSM13	19.4	60	161 ±7	√	●	[●]	○	○	○
S-RSM14	4.10	80	157 ±9	√	●	[●]	○	○	○
S-RSM15	19.4	10	156 ±3	√	[●]	●	○	○	○
S-RSM16	11.5	125	151 ±6	√	●	[●]	○	○	○
S-RSM17	19.4	80	160 ±3	√	[●]	○	○	○	○
S-RSM18	11.5	50	157 ±3	√	●	[●]	○	○	○
S-RSM19	27.4	80	165 ±6	√	●	[●]	○	○	○
S-RSM20	4.10	50	162 ±8	√	●	[●]	○	○	○
S-RSM21	18.10	100		√	●	[●]	○	○	○
S-RSM22	7.9	125	152 ±1	√	●	[●]	○	○	○
S-RSM23	18.10	125		√	●	[●]	●	○	○
S-RSM24	11.5	100	154 ±2	√	●	[●]	○	○	○
S-RSM25	27.4	60	155 ±3	√	●	[●]	○	○	○
S-RSM26	27.4	50	154 ±9	√	●	[●]	○	○	○
S-RSM27	18.10	125		√	●	[●]	○	○	○
S-RSM28	11.5	50	154 ±10	√	●	[●]	○	○	○
S-RSM29	19.4	30	152 ±6	√	●	[●]	○	○	○
S-RSM30	4.10	50	163 ±3	√	●	[●]	○	○	○
S-RSM31	18.1	125		√	●	[●]	○	○	○
S-RSM32	19.4	60	152 ±3	√	●	[●]	○	○	●
S-RSM33	18.10	125		√	●	[●]	●	○	○
S-RSM34	27.4	60	151 ±4	√	●	[●]	○	○	○
S-RSM35	27.4	80	152 ±3	√	●	[●]	○	○	○
S-RSM36	18.10	125	156 ±4	√	●	[●]	○	○	○
S-RSM37	22.11	150	175 ±6	√	●	[●]	●	○	○
S-RSM38	27.4	60		√	●	[●]	○	○	○
S-RSM39	27.4	30	192 ±5	√	●	[●]	○	○	○
S-RSM40	19.4	60	197 ±6	√	[●]	●	○	○	○
S-RSM41	19.4	60	194 ±4	√	[●]	●	○	○	○
S-RSM42	19.4	80	162 ±10	√	[●]	●	○	○	●
S-RSM43	27.4	50	189 ±7	√	[●]	○	○	○	○
S-RSM44	27.4	30		√	[●]	●	○	○	●
S-RSM45	27.4	80	154 ±3	√	[●]	●	○	○	●
S-RSM46	27.4	80	160 ±9	√	[●]	○	○	○	○
S-RSM47	19.4	30		√	[●]	●	○	○	○
S-RSM48	11.5	50	151 ±2	√	[●]	●	○	○	○
S-RSM49	11.5	50	158 ±5	√	[●]	●	●	○	○
S-RSM50	27.4	80	159 ±3	√	[●]	○	○	○	○
S-RSM51	11.5	80	153 ±3	√	[●]	●	○	○	○
S-RSM52	11.5	80	192 ±1	√	[●]	●	○	○	○
S-RSM53	11.5	80	167 ±6		[●]	●	○	○	○
S-RSM54	11.5	80		√	[●]	○	○	○	●
S-RSM55	11.5	80	185 ±13	√	[●]	●	○	○	○
S-RSM56	11.5	80	153 ±6	√	[●]	●	○	○	●
S-RSM57	11.5	100		√	[●]	○	○	○	●
S-RSM58	11.5	150	152 ±8	√	[●]	●	○	○	○
S-RSM59	14.6	60	150 ±4	√	[●]	●	○	○	○

Continued opposite

Table 1. *Continued.*

Phage	Date of isolation	Depth	Genome size	Gp20 product	Host range WH7803	WH8103	RS9906	RS9911	SS120
S-RSM60	18.7	30		√	[●]	●	○	○	○
S-RSM61	18.7	30		√	[●]	●	○	○	○
S-RSM62	18.7	150	179 ±6	√	[●]	●	○	○	○
S-RSM63	23.8	80		√	[●]	○	○	○	○
S-RSM64	7.9	30	156 ±6	√	[●]	●	○	○	○
S-RSM65	7.9	80	167 ±3		[●]	●	○	○	○
S-RSM66	18.10	125	153 ±6	√	[●]	●	●	○	○
S-RSM67	18.10	125		√	[●]	●	●	●	○
S-RSM68	18.7	150		√	[●]	○	○	○	○
S-RSM69	18.7	125	189 ±13	√	[●]	●	○	○	○
S-RSM70	23.8	10	153 ±3		[●]	●	○	○	○
S-RSM71	18.10	30		√	[●]	○	○	○	○
S-RSM72	18.7	10	197 ±11	√	[●]	○	○	○	○
S-RSM73	27.12	30	179 ±4	√	[●]	○	○	○	○
S-RSM74	11.5	80	156 ±10	√	[●]	●	○	○	○
S-RSM75	27.4	30	151 ±14	√	[●]	●	○	○	○
S-RSM76	19.4	125	183 ±6	√	[●]	●	○	○	○
S-RSM77	11.5	50	154 ±4.4	√	[●]	●	○	○	○
S-RSM78	18.7	150	180 ±6		[●]	○	○	○	○
S-RSM79	18.10	80		√	[●]	○	○	○	○
S-RSM80	11.5	80	185 ±16	√	[●]	●	○	○	○
S-RSM81	19.4	80	151 ±3	√	[●]	●	○	○	○
S-RSM82	11.5	80	195 ±13	√	[●]	●	○	○	○
S-RSM83	19.4	30			[●]	○	○	○	○
S-RSM84	19.4	125	186 ±12	√	[●]	●	○	○	○
S-RSM85	19.4	125	189 ±8	√	[●]	○	○	○	○
S-RSM86	19.4	125	201 ±4	√	[●]	○	○	○	○
S-RSM87	19.4	125	204 ±7	√	[●]	○	○	○	○
S-RSM88	19.4	125	155 ±3	√	●	○	[●]	○	○
S-RS89*	19.4	125	198 ±16	√	[●]	○	○	○	○

*, This phage was lost before a complete characterization could be completed; [●], Strain used for isolation; ●, Phage produced plaques/clearing of wells; ○, No plaques produced/no clearing of wells; √, A gp20 product was obtained using the primers CPS-1 and CPS-2.

placed in a lysis buffer of 0.1 M EDTA, 1% SDS, 0.1 M Tris−HCl pH 9.0, 0.5 mg ml^{-1} proteinase K and incubated at 55°C overnight. The plugs were washed twice with 200 mM TE, and stored at 4°C until ready for use. Samples were run in a 1% PFGE-grade agarose gel (Bio-Rad) with lambda PFGE ladders (Sigma) run in the outside lanes. Gels were run initially in a CHEF Mapper (Bio-Rad) in 0.5 × TBE at 14°C, 6 V cm^{-1} with an initial switch time of 6.75 s and a final switch time of 21.79 s for 20 h. After it became apparent that no isolate had a genome size of less than 100 kb the switch times were changed to an initial switch time of 12.55 s and a final switch time of 17.33 s, all other conditions were kept constant. Gels were stained with ethidium bromide (0.5 $\mu g\,l^{-1}$) for 1 h and then washed in deionized H_2O for 20 min. Gel images were digitally captured using a Syngene gel documentation system (Cambridge, UK). A genome size for each phage was estimated by taking the average genome size from a minimum of three replicates.

RESULTS

Cyanophage abundance

It was only possible to enumerate phages from April 1999 to December 1999 and this period will be referred to as the annual cycle, although it does not cover a full 12 months. Phage abundance was determined using four *Synechococcus* strains, but phage numbers were found to be highest when *Synechococcus* sp. WH7803 was used as a host. Cyanophages were found at all depths throughout the annual cycle, but were not found using all strains of *Synechococcus* as hosts. Titres of cyanophages ranged from undetectable to 6.08×10^3 pfu ml^{-1} (Figure 1A–K).

For the months of April, May and June the abundance of cyanophages was low with often no phages detected at some depths when strains WH8103, RS9906 and RS9911 were used as hosts (Figure 1A–D). In July an increase in phage number was found at 10 m and a maximum in the number of phages infecting *Synechococcus* sp. WH7803 was observed at a depth of 30 m (Figure 1E). It was at this depth that the highest cyanophage abundance (6.08×10^3 pfu ml^{-1}) of the entire annual cycle was observed in September (Figure 1G). This pattern was not observed when RS9906, RS9911 and WH8103 were used to estimate phage abundance (Figure 1E–G). The high abundance of phage at 30 m continued into August and September, but by early October abundance had started to decline and by late October the decline was complete (Figure 1F–I). During August, September and early October there was a small increase in phage numbers,

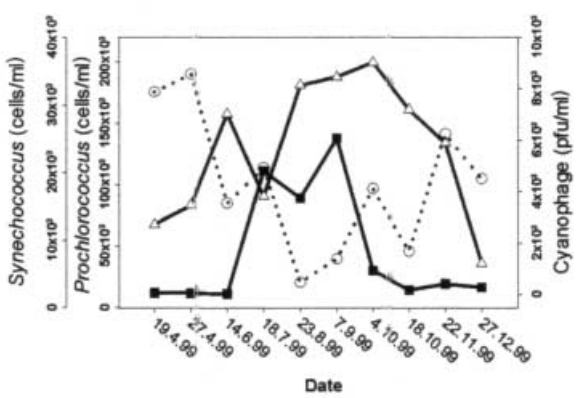

Figure 2. *Synechococcus*, *Prochlorococcus* and cyanophage abundance during the annual cycle in the Gulf of Aqaba at a depth of 30 m. Cyanophage numbers are representative of those obtained using *Synechococcus* sp. WH7803 as a host (■). *Synechococcus* (○) and *Prochlorococcus* (△) numbers were from flow cytometry counts from previously published data (Fuller et al., 2005).

most notably at a depth of 150 m in August and September (Figure 1G,H).

Using *Synechococcus* strains WH8103, RS9906 and RS9911 as hosts, far lower phage titres were obtained (Figure 1A–K). Phages capable of infecting these strains were detected throughout the year in very low abundances, however, the highest numbers of phage found were in late October, November and December. Host strains RS9906 and RS9911 revealed a similar profile of phage abundance, with maxima at 50 m in November and December (Figure 1J,K). When WH8103 was used as a host the maximum in phage abundance was found at a depth of 100 m (Figure 1J). However, this peak of 5×10^2 pfu ml^{-1} was an order of magnitude lower than the titre of phages found to infect WH7803 at a depth of 30 m in July.

The abundances of cyanophages and their potential *Synechococcus* hosts at 30 m do not covary (Figure 2). When *Synechococcus* numbers are at their highest during the months of November, December and April the numbers of cyanophages were at their lowest. Using Pearson's correlation it was found there was no significant correlation between the abundance of cyanophages and *Prochlorococcus* (-0.51) or *Synechococcus* (0.33) for the entire annual cycle. However, it should be noted that the maxima in cyanophage abundance during July, August and September coincide with maximal *Prochlorococcus* abundance (Figure 2).

Host range

The host used for cyanophage enumeration was found to be important in estimating cyanophage numbers. Of all the plaques counted, 88% were observed on WH7803 plates. With 6%, 4% and 2% of total plaques found when using RS9906, WH8103 and RS9911 as hosts respectively. A total of 87 cyanophages was isolated using different hosts and were characterized in terms of their ability to infect the other strains of *Synechococcus* used in estimating phage abundance. It was found that ~98% of phage were capable of infecting *Synechococcus* WH7803 and 75% capable of infecting WH8103 (Table 1). Far fewer phages could infect the other two strains used, with ~9% and ~1% capable of infecting RS9906 and RS9911 respectively (Table 1). The ability of phage isolates to infect multiple hosts was found to be widespread with ~77% of phage infecting at least two strains. However, only phage S-RSM67 was capable of infecting all four strains (Table 1). The host range of isolates was correlated with the strain used for isolation. Sixty-five per cent of the phages isolated on WH7803 could infect WH8103, whereas of the phages isolated on WH8103 ~91% could also infect WH7803 (Table 1).

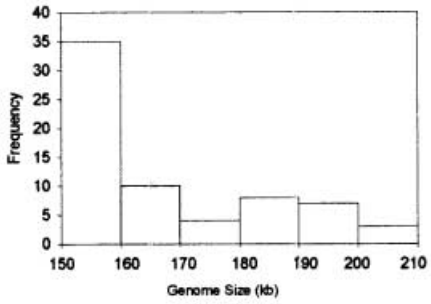

Figure 3. Histogram of the genome size of 67 cyanophages isolated from the Red Sea.

The coincidence of the maxima in cyanophage abundance during July, August and September with maximal *Prochlorococcus* abundance (Figure 2) suggests that cyanophages being titred on *Synechococcus* may also be capable of infecting *Prochlorococcus* strains. To test this hypothesis the 87 Red Sea cyanophage isolates, S-PM2, and S-WHM1 were screened for their ability to infect *Prochlorococcus* strain SS120. It was found for the phages S-WHM1, S-RS11, S-RS33, S-RS42, S-RS44, S-RS45, S-RS54, S-RS56 and S-RS57 could cause the clearing of cultures of *Prochlorococcus* (Table 1) and therefore might be assumed to be infective. However, it was not possible to determine whether this was true infection and lysis or some phenomenon like lysis from without, where a number of phage absorb to and lyse a cell without the production of progeny phage (Delbruck, 1940). In the case of phage S-WHM1, the titre of phage added to the *Prochlorococcus* was 2.64×10^6 pfu ml^{-1} using *Synechococcus* sp. WH7803 as a host, but after apparent lysis the supernatant had a phage titre of only 1.67×10^6 pfu ml^{-1}.

Cyanophage classification

A total of 87 cyanophages were isolated from samples obtained at different times and depths through the annual cycle. The phage isolates were screened by PCR using the cyanomyovirus-specific primers CPS-1 and CPS-2 (Fuller et al., 1998). When a correctly sized 165 bp PCR product was obtained the phage in question was classified as a cyanomyovirus. A negative result was taken to be inconclusive, as the primers are known not to amplify g20 from all cyanomyoviruses (Fuller et al., 1998).

It was possible to identify 82/87 isolates as being members of the Myoviridae family by using this PCR approach (Table 1). The remaining unidentified isolates RS53, RS65, RS70, RS78 and RS83 were examined by TEM to try to determine which family of viruses they belong to. In each case the phages were found to have

tails of variable lengths, suggesting that the tail is contractile, and therefore that the phage is a member of the Myoviridae family. Thus all the isolates were identified as cyanomyoviruses.

Genome size

It was possible to determine the genome size of 67 of the 87 cyanophage by the use of PFGE. The cyanophage S-PM2 was used as control as it has previously been estimated to have a genome size of 194 kb by PFGE (Hambly et al., 2001) and is now known to have an actual genome size of 196.28 kb (Mann et al., 2005). In this study S-PM2 was estimated to have the genome size of 197 ±4 kb indicating that PFGE was yielding an accurate estimation of genome size. The smallest estimated genome size in this study was that of cyanophage S-RSM59, which had a genome size of 150 ±4 kb and the largest had genomes in excess of 200 kb (Figure 3).

DISCUSSION

Previous studies have examined cyanophage numbers over an annual cycle, and found that cyanophage numbers are not constant over the period. In a study of cyanophage numbers from March 2001 to October 2002 in Mt Hope Bay, Rhode Island, it was found there was a seasonality to cyanophage numbers (Marston & Sallee, 2003). This seasonal variation was partly attributed to the variation in *Synechococcus* abundance, as previous studies have found that cyanophage abundance is closely related to *Synechococcus* abundance (Waterbury & Rippka, 1989; Lu et al., 2001). In a study in the Gulf of Mexico it was found that phage abundance was closely linked to *Synechococcus* abundance, with a threshold value of $10^3\,ml^{-1}$ *Synechococcus* cells, above which phage numbers increased from 10^2 to $10^5\,ml^{-1}$ (Suttle & Chan, 1994). In this study where cyanophage numbers increased 100-fold from June to July at a depth of 30 m (Figure 2), the total number of *Synechococcus* has been found to decrease, but it did stay above the threshold value of 10^3 cells ml^{-1} (Fuller et al., 2005). This decrease in *Synechococcus* numbers and increase in cyanophage is in contradiction to other reports of cyanobacteria/phage relationships (Waterbury & Valois, 1993; Sullivan et al., 2003). It is important to realize that the decrease in *Synechococcocus* numbers is only representative of the total *Synechococcus* population as determined by flow cytometer counts. These counts are unable to detect any subtle changes in *Synechococcus* populations. Therefore, the increase in a population of hosts susceptible to infection could be masked by the net decrease in the abundance of all other *Synechococcus* populations.

There is also the possibility that there is a host other than *Synechococcus* that the cyanophages can infect. It was observed that although *Synechococcus* numbers decreased during the cyanophage maxima in the period from July to September, the number of *Procholorococcus* was still very high (Figure 2). It is possible that cyanophages capable of infecting both *Synechococcus* and *Procholorococcus* were being detected. It is known that some cyanophages can infect both *Synechococcus* and *Procholorococcus* (Sullivan et al., 2003). A parallel study using the same water samples revealed that the genetic diversity of *Prochlorococcus* changed over the same annual cycle (Fuller et al., 2005). Using 16S rRNA dot blot hybridization high-light (HL)-*Prochlorococcus* genotypes were found to dominate from April to December and low-light (LL)-*Prochlorococcus* were found from July to early October. By mid-October LL-*Prochlorococcus* genotypes were not detected. This observation is important as Sullivan et al. (2003) found cyanophages that were able to infect both *Synechococcus* and LL-*Prochlorococcus*, but not *Synechococcus* and HL-*Prochlorococcus* (Sullivan et al., 2003). The increase in LL-*Prochlorococcus* in the Red Sea during the month of August also coincided with an increase in the abundance of cyanophages at a depth of 30 m (Figure 2); the decrease by mid October also coincided with the decrease in phage numbers at 30 m. The hypothesis that cyanophages may infect *Prochlorococcus* was tested by attempting to infect *Prochlorococcus* strain SS120 using the 87 Red Sea cyanophage isolates, together with phages S-PM2 and S-WHM1 as negative and positive controls respectively. It was found that several phage isolates, including S-WHM1, could cause the clearing of cultures of *Procholorococcus*. However, it was not possible to determine whether this was directly caused by the addition of phage leading to infection and subsequent lysis. In the case of phage S-WHM1 there was no increase in phage titre after lysis of the *Prochlorococcus* culture, as measured using *Synechococcus* sp. WH7803 as a host. This could suggest that S-WHM1 does not infect *Prochlorococcus* sp. SS120, which would be in conflict with a previous report (Sullivan et al., 2003). Alternatively, it could be that after infection of *Prochlorococcus* the progeny phage lacked the ability to efficiently infect *Synechococcus* as a result of altered DNA modification or the production of phage virions with alternative adhesins. However, it was not possible to distinguish between these possibilities without the ability to grow *Prochlorococcus* on agar plates and detect individual plaques.

Host range

It was apparent that the number of cyanophages that were detected is dependent on the host strain of *Synechococcus* used for enumeration (Figure 1A–K). A similar observation was made previously by Waterbury & Valois (1993) who found that cyanophage numbers varied from 3.5×10^1 when using *Synechococcus* sp. WH8108 as a host compared to 7×10^3 with host strain *Synechococcus* sp. WH8017. Lu et al. (2001), also found that on average phage numbers were 324 times higher using *Synechococcus* sp. WH7803 as a host compared to *Synechococcus* sp. WH8101.

As *Synechococcus* sp. WH7803 gave the highest estimation of phage abundance (Figure 1) it was unsurprising that the majority of phages were capable of infecting the *Synechococcus* WH7803 in the host range studies (Table 1). However, ~75% of phage isolates were found to infect WH8103, yet the abundance of phages estimated with WH8103 used were significantly lower than when WH7803 was used as host for phage enumeration.

Host range has previously been linked to phage morphology. For example, phages that infect HL-*Prochlorococcus* have been shown to be exclusively podoviruses and host specific, while myoviruses could infect multiple strains of *Synechococcus* or *Prochlorococcus* and some infect both genera (Sullivan et al., 2003). All phages isolated in this study are thought to be

myoviruses, yet it was found that some phages such as S-RSM12 could infect only one strain while others such as S-RSM67 could infect all fours strains of *Synechococcus* tested (Table 1).

The host range of cyanophages have previously been studied in some detail (Suttle & Chan, 1993, 1994; Waterbury & Valois, 1993; Wilson et al., 1993; Lu et al., 2001). Marine unicellular cyanobacteria possessing phycoerythrin as their primary accessory light-harvesting pigment were originally classified as marine cluster A (MC-A) *Synechococcus* and distinguished from marine cluster B (MC-B), members of which have phycocyanin as their major light-harvesting pigment (Waterbury & Rippka, 1989). The recent division of the genus *Synechococcus* into five clusters (Herdman et al., 2001) makes the comparison with previous studies difficult. Marine *Synechococcus* now comprise sub-clusters 5.1 and 5.2. The new sub-cluster 5.1 is representative of the majority of what was previously MC-A and sub-cluster 5.2 represents the majority of what was MC-B. For the purpose of comparison with previous studies, strains in this study will still be referred to as MC-A or MC-B. The MC-A strains can be divided into ten clades by the phylogenetic analysis of 16S rDNA (Fuller et al., 2003). All strains used in this study are MC-A strains, with *Synechococcus* sp. WH7803 a representative of clade V, *Synechococcus* sp. WH8103 a representative of clade III, *Synechococcus* sp. RS9906 a representative of clade VIII and *Synechococcus* sp. RS9911 a representative of clade II. These four clades are monophyletic and have all evolved from a common ancestral host. Clades VIII and III show greater evolutionary distance from the common ancestor than clade II and clade V (Fuller et al., 2003) . It therefore might be expected that a phage is more likely to infect both a clade II and clade V *Synechococcus* than a clade II and clade VIII *Synechococcus*.

The host range of cyanophages, in common with phage morphology, has previously been shown to be linked to the strain of *Synechococcus* used for isolation. Suttle & Chan (1994) isolated phages infecting a MC-A *Synechococcus* and a green-coloured strain (possibly MC-B) of *Synechococcus*. They found that there was greater cross infection between MC-A strains than between MC-A strains and the green strain of *Synechococcus*. The results of this study (Table 1) indicate that there is greater cross infection between WH7803 (clade V) and WH8103 (clade II) than between RS9906 (clade VIII) and WH7803 (clade V) or RS9906 (clade VIII) and WH8103 (clade III). These results were similar to that of Suttle & Chan (1993) as both WH7803 and WH8103 are orange/red-coloured where as RS9906 is a green-coloured strain of *Synechococcus*. The comparison with previous studies is made difficult as there is no genetic data available for all strains used in previous studies, so it cannot be determined what genotype a strain may be. New observations contrary to previous studies (Suttle & Chan, 1993) were found in this study, with only two of the isolated phages infecting the clade II strain *Synechococcus* sp. RS9911 (also a red/orange-coloured strain), in contrast to the eight that infected the green RS9906 (clade VIII strain). The results therefore indicate that more phages do not cross infect *Synechococcus* from clade II and VIII as might be expected but show a greater cross infectivity between clade V (WH7803) and clade III (WH8103). As current *Synechococcus* phylogeny is based on 16S rDNA, it is unlikely to be useful in the prediction of which strains of *Synechococcus* a phage can infect, as there will be no direct selective pressure on the 16S rDNA of *Synechococcus* by a phage. Whereas the phylogeny of receptors on the host are much more likely to predict what phage a host can infect. However, until the receptors on the host are identified and the tail fibres in phages are found, the ability of phages to infect multiple strains of *Synechococcus* is unlikely to be fully explained.

The ability of the cyanophages in this study to infect multiple hosts, also fits well with the theory of Wommack & Colwell (Wommack & Colwell, 2000), that phage from oligotrophic environments have broader host ranges. The thinking behind this hypothesis is that phage from oligotrophic water will encounter hosts less often, and will therefore have an advantage if they can infect more than one host, as they are more likely to encounter a host they can infect.

It has been proposed that the differences in the host ranges of cyanophages will be influenced by contact rates between host cells and phage. When contact rates are high, there will be a strong selection for resistant cells; when contact rates are lower there will be less selection pressure for resistant hosts (Waterbury & Valois, 1993; Suttle & Chan, 1994). In the Red Sea, clade II strains of *Synechococcus* have been found to dominate throughout an annual cycle and RS9911 is a representative of this clade (Fuller et al., 2003). It could be that RS9911 and its relatives are resistant to most of the co-occurring phages and this allows their dominance. However, no studies have yet systematically looked at patterns of phage resistance within a clade.

This study has shown that in an oligotrophic body of water representative of an open ocean system there is seasonal variation in cyanophage numbers as was previously found in coastal studies (Suttle & Chan, 1993; Waterbury & Valois, 1993; Marston & Sallee, 2003). However, it has also revealed the difficulty with currently available techniques of identifying the true impact of phage infection on natural marine cyanobacterial communities. It is impossible to assess the total infectivity of phages present in a given environment with respect to a complex community of potential hosts and the potential phage:host interactions are complicated by abiotic variables such as nutrient availability, light intensity etc. A further convolution is the impact of grazers on this system. Future studies on the nature of the phage receptors on the surface of *Synechococcus* and *Prochlorococcus* cells and phage adhesins may provide us with both an understanding of these phage:host interactions and the molecular tools with which to quantify them *in situ*.

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