

ORIGINAL ARTICLE

Comparison of lysogeny (prophage induction) in heterotrophic bacterial and *Synechococcus* populations in the Gulf of Mexico and Mississippi river plume

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Lysogeny has been documented as a fundamental process occurring in natural marine communities of heterotrophic and autotrophic bacteria. Prophage induction has been observed to be prevalent during conditions of low host abundance, but factors controlling the process are poorly understood. A research cruise was undertaken to the Gulf of Mexico during July 2005 to explore environmental factors associated with lysogeny. Ambient physical and microbial parameters were measured and prophage induction experiments were performed in contrasting oligotrophic Gulf and eutrophic Mississippi plume areas. Three of 11 prophage induction experiments in heterotrophic bacteria (27%) demonstrated significant induction in response to Mitomycin C. In contrast, there was significant *Synechococcus* cyanophage induction in seven of nine experiments (77.8%). A strong negative correlation was observed between lysogeny and log-transformed activity measurements for both heterotrophic and autotrophic populations ($r = -0.876$, $P = 0.002$ and $r = -0.815$, $P = 0.025$, respectively), indicating that bacterioplankton with low host growth favor lysogeny. Multivariate statistical analyses indicated that ambient level of viral abundance and productivity were inversely related to heterotrophic prophage induction and both factors combined were most predictive of lysogeny ($\rho = 0.899$, $P = 0.001$). For *Synechococcus*, low ambient cyanophage abundance was most predictive of lysogeny ($\rho = 0.862$, $P = 0.005$). Abundance and productivity of heterotrophic bacteria was strongly inversely correlated with salinity, while *Synechococcus* was not. This indicated that heterotrophic bacterial populations were well adapted to the river plume environments, thus providing a possible explanation for differences in prevalence of lysogeny observed between the two populations.

The ISME Journal (2008) 2, 132–144; doi:10.1038/ismej.2007.102; published online 29 November 2007

Subject Category: microbial population and community ecology

Keywords: FVIC; lysogeny; prophage induction; river plume; *Synechococcus*

Introduction

Lytic viral infections have been estimated to contribute to about half the bacterial mortality each day in aquatic environments. These lytic processes provide an important source of nutrients, especially organic carbon for microbial growth. Recent research has indicated that up to 83% of the organic carbon released due to lysis is immediately recycled by bacteria (Middelboe and

Jørgensen, 2006). Besides constraining the overall abundance of bacteria, evidence is mounting that bacteriophages can influence bacterial diversity in the marine environment, including seasonal succession of populations (Wommack and Colwell, 2000; Weinbauer and Rassoulzadegan, 2004; Mühling *et al.*, 2005; Suttle, 2005; Hewson *et al.*, 2006).

Previous research has indicated that viruses are unable to persist for long periods of time in the water column, especially in the highly productive photic zone where ultraviolet light is the primary basis for their decay (Noble and Fuhrman, 1997). An important potential reservoir of some bacteriophages may be found in lysogenic bacteria. Prophage can be stable in their host for long periods of time with low levels of bacteriophage being released

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Received 1 May 2007; revised 13 October 2007; accepted 22 October 2007; published online 29 November 2007

by spontaneous lysis. Large numbers of phage can also be produced by prophage induction resulting from encountering a mutagen, or responding to favorable growth conditions that favor host lysis. Recent research has indicated that such prophage induction events change bacterial community structure by increasing the diversity and richness of natural microbial communities (Hewson and Fuhrman, 2007). As such, it is important to investigate factors that may lead to induction of natural prophage populations.

It has long been postulated that lysogeny is a survival strategy allowing viruses to persist during periods of low host availability or poor growth conditions. Lysogeny has been experimentally verified to be widespread among cultured bacterial isolates (Ackermann and DuBow, 1987; Ackermann, 2003). The occurrence of lysogeny in cultured marine bacteria as well as natural populations has been well documented, although it tends to be temporally and spatially variable, being more prevalent during the winter months (Jiang and Paul, 1994, 1996; Williamson *et al.*, 2002). This seasonality has been observed in environments as divergent as the Antarctic and the subtropical Gulf of Mexico (McDaniel *et al.*, 2002; Williamson *et al.*, 2002; Laybourn-Parry *et al.*, 2007).

Lysogeny has also been documented in the important photosynthetic microbe *Synechococcus* (McDaniel *et al.*, 2002; Ortmann *et al.*, 2002). An examination of prophage induction in cultured *Synechococcus* isolates from the Gulf of Mexico indicated that about half of the isolates contained inducible prophage-like particles, in agreement with previous estimates for heterotrophic bacteria (McDaniel *et al.*, 2006). In experiments with natural populations, *Synechococcus* prophage induction (cyanophage induction) was not generally found to be nutrient limited. In these studies, the most important factors associated with the prevalence and level of induction were the ambient levels of infectious cyanophage and *Synechococcus* (McDaniel and Paul, 2005). This study also suggested that the level of system productivity was most likely a related factor, yet the data were insufficient to provide conclusive support for this hypothesis.

In this paper, we examined two null hypotheses regarding lysogeny in natural populations of heterotrophic bacteria and marine phycoerythrin-containing *Synechococcus*. The first hypothesis was that there is no relationship between lysogeny and the level of productivity in an environment. The second hypothesis was that there would be no detectable difference between the prevalence and/or environmental factors associated with prophage induction in heterotrophic and autotrophic marine bacterial populations. Our testing grounds for these hypotheses were the hypertrophic Mississippi River plume (MRP) and adjacent oligotrophic waters of the Gulf of Mexico.

Materials and methods

Sample collection

A research cruise was conducted during July 2005 to investigate lysogeny in strongly contrasting trophic environments, the MRP and the oligotrophic Gulf of Mexico. Surface samples were collected at a 3 m depth using a submersible pump. Subsurface samples were collected using 5 l Niskin bottles mounted on a SeaBird SBE CTD/Rosette system with sensors for temperature, salinity, oxygen photosynthetically active radiance (PAR) and fluorescence. All samples were pre-filtered with 50 μm mesh screen to remove macro-organisms.

In total, eight stations were sampled, three of which were in oligotrophic waters and the remaining five were located in areas from the proximal to distal MRP. The depths sampled at each station varied. The letter following the station number indicates the depth or time point sampled (Table 1). At station 1, there were two surface samples and two subsurface samples, one at the subsurface chlorophyll maximum (SCM), which was at 60 m and the other at 500 m. Station 2 was the oligotrophic depth profile; eight depths were sampled at the surface, above the SCM, the SCM at 88 m, and below. A depth profile of eutrophic waters was done at station 3 where four depths, including the surface, were sampled. Stations 4, 5 and 6 were all plume stations and were surface stations only. At station 7, a free-floating marker buoy was placed in the water allowing for Lagrangian surface sampling of a discrete parcel of the distal plume every 4 h for 24 h resulting in a total of six samples for that station. The starting time was at 18:41 on July 17 and measurements were taken every 4 h until 14:33 on 18 July. At a final oligotrophic station, station 8, two samples were taken, one at the surface and the other at the SCM (55 m).

Enumeration of bacteria and viruses

Heterotrophic bacterial and viral populations were enumerated by filtering an appropriate sample volume onto 0.02 μm 25 mm Anodisc filters (Whatman, Maidstone, England) followed by staining with Sybr Gold nucleic acid stain at a 1:10 000 final dilution (Molecular Probes, Eugene, OR, USA); (Noble and Fuhrman, 1998; Chen *et al.*, 2001). Slides were made in triplicate. The volume filtered was dependent on the sample location and depth, and ranged from 2 ml (3 m) to 8 ml (140–500 m). Filters were stained for 12 min in the dark and mounted with an anti-fade solution composed of p-phenyldiamine in 50/50 filter-sterilized glycerol and phosphate-buffered saline. To provide the most accurate enumeration the slides were prepared immediately, tightly wrapped in foil, stored at -20°C and counted within 1 month after completion of the cruise (Wen *et al.*, 2004). Samples were counted using an epifluorescence microscope with

Table 1 List of the ambient parameters measured for all cruise stations

Station	Depth (m)	Temperature (°C)	Salinity (p.s.u.)	Total bacteria (cells ml ⁻¹)	s.d. (cells ml ⁻¹)	Total virus (VLPs ml ⁻¹)	s.d. (VLPs ml ⁻¹)	Synechococcus (cells ml ⁻¹)	s.d. (cells ml ⁻¹)	Synechococcus cyanophage (infectious cyanophage ml ⁻¹)	s.d. (infectious cyanophage ml ⁻¹)	Primary production (mg C liter ⁻¹ h ⁻¹)	Bacterial production (pM leucine liter ⁻¹ h ⁻¹)	Frequency of infected cells (%)	Lysogenic fraction (percentage of total)
<i>Oligotrophic</i>															
1A	3	27.2	35.9	6.36E+05	4.91E+04	6.90E+06	4.53E+05	5.31E+03	2.6E+03	7.70E+02	3.7E+02	ND	32.4	0	-0.039
1B	60	22.2	36.36	4.52E+05	3.56E+05	4.60E+06	3.75E+06	2.49E+03	1.2E+03	9.27E+02	1.9E+02	ND	12.82	4.1	-0.134
1XS	3	28.9	36.1	5.98E+05	5.53E+04	5.99E+06	1.52E+05	7.24E+03	2.3E+03	1.09E+03	5.0E+02	ND	45.2	4.1	0.225
1XD	500	9	35	6.55E+04	9.43E+03	5.87E+05	1.51E+05	ND	ND	0	0.0E+00	ND	0	0.7	6.64
8A	3	28.64	35.67	7.79E+05	1.40E+05	7.43E+06	5.33E+05	1.07E+04	1.4E+03	1.77E+02	1.1E+01	0.6967	66.6666	0.69	-1.05
8B	55	23	36.27	1.41E+06	1.04E+05	1.37E+07	5.63E+05	1.22E+03	2.9E+02	9.66E+02	3.8E+02	0.4377	30.9738	0.7	2.52
(SCM)															
2A	3	29.5	34.2	9.46E+05	2.31E+05	1.64E+07	2.74E+06	3.01E+04	7.1E+02	2.47E+04	6.3E+03	0.9877	83.0544	2.4	-2.02
2B	30	25.32	35.86	6.20E+05	1.39E+05	6.15E+06	2.40E+06	6.12E+03	3.0E+03	1.79E+04	1.9E+04	0.9896	24.74472	ND	ND
2C	40	25.48	36.4	9.11E+05	5.82E+04	7.11E+06	1.13E+06	3.26E+03	2.5E+03	3.61E+03	1.3E+03	1.3905	30.74214	ND	ND
2D	50	24.58	36.24	7.00E+05	6.58E+04	8.11E+06	8.81E+05	1.56E+03	1.8E+03	5.51E+03	1.0E+03	2.5516	71.93472	ND	ND
2E	76	21.2	36.45	7.69E+05	5.13E+04	1.00E+07	6.49E+05	1.31E+02	9.7E+01	2.72E+02	6.4E+01	0.2508	2.2308	ND	ND
2F	88	20.7	36.5	2.95E+05	1.64E+04	5.77E+06	6.39E+05	2.27E+02	4.3E+01	5.01E+02	1.7E+02	0.2007	0.86658	0.65	6.94
(SCM)															
2G	100	20.1	36.5	2.75E+05	1.80E+04	4.41E+06	2.86E+05	1.21E+02	1.9E+01	5.23E+01	1.6E+01	0.0894	0.32604	ND	ND
2H	140	17.6	36.57	1.72E+05	7.86E+04	1.62E+06	8.04E+05	6.00E+01	1.0E+01	4.60E+01	1.2E+01	ND	1.48434	ND	ND
<i>Plume</i>															
3A	3	29.14	30.9	3.05E+06	2.28E+05	3.66E+07	1.23E+06	2.32E+04	8.3E+03	5.61E+04	5.4E+03	4.5129	451.9944	ND	ND
3B	8	28.95	31.4	2.32E+06	3.99E+05	1.95E+07	1.17E+07	2.32E+04	4.0E+03	8.69E+04	2.9E+04	3.1911	807.807	ND	ND
(SCM)															
3C	35	25.41	35.8	1.11E+06	8.43E+04	9.54E+06	4.09E+05	2.04E+03	1.4E+02	6.34E+03	2.1E+03	0.5313	10.97382	ND	ND
3D	100	19.45	36.4	4.82E+05	5.80E+04	3.06E+06	4.76E+05	4.28E+02	3.9E+01	2.04E+02	5.0E+01	0.3500	3.5178	ND	ND
4A	3	29.39	30.12	3.31E+06	1.73E+05	3.71E+07	4.04E+06	2.41E+04	3.0E+03	4.23E+04	4.9E+03	7.1467	661.518	7.05	0.649
5A	3	28.5	31.98	2.64E+06	2.09E+05	3.45E+07	3.25E+06	1.08E+05	3.3E+04	1.08E+05	3.2E+04	6.9084	517.1166	ND	ND
6A	3	29.07	31.6	2.66E+06	1.92E+05	3.17E+07	3.38E+06	1.34E+05	2.1E+03	9.49E+04	7.0E+03	2.8128	295.8384	ND	ND
7A	3	29.1	31.32	3.27E+06	7.04E+05	3.53E+07	6.69E+05	1.28E+05	9.0E+03	9.36E+04	3.4E+04	5.9369	540.54	ND	ND
7B	3	28.977	31.4	4.34E+06	3.45E+05	3.02E+07	1.91E+06	4.12E+04	3.8E+03	8.37E+04	2.0E+04	2.8112	641.784	ND	ND
7C	3	28.95	31.5	2.98E+06	1.41E+05	2.92E+07	7.92E+05	1.29E+05	4.0E+04	1.21E+05	4.0E+04	4.9414	386.958	ND	ND
7D	3	28.85	31.99	2.57E+06	3.10E+05	2.84E+07	8.50E+06	1.81E+05	1.4E+04	1.88E+05	6.6E+04	0.7566	425.568	ND	ND
7E	3	28.864	31.99	1.41E+06	1.31E+05	1.61E+07	1.50E+06	3.26E+04	8.7E+03	1.41E+05	7.7E+03	5.7069	471.042	ND	ND
7F	3	29.24	31.81	3.25E+06	1.55E+05	3.31E+07	3.15E+06	1.25E+05	5.7E+03	2.46E+05	3.5E+04	2.3756	438.438	3.15	-0.798
7G	67	23.2	36.5	4.27E+05	8.49E+04	6.36E+06	2.23E+06	1.65E+03	3.3E+01	9.10E+01	3.8E+01	0.1749	6.82968	3.32	1.68
(SCM)															

Abbreviations: ND, not done; s.d., standard deviation.

The stations are grouped by trophic level. Letters following the station numbers differentiate the different depths or sampling times when more than one sample was obtained from that station. Subsurface samples are shaded in gray to differentiate them from surface samples.

blue excitation. Bacteria and viruses were on the same filter and were differentiated by size and fluorescence intensity. The ratio of viruses to bacteria (VBR) was calculated as an indicator for host–viral interactions for the ambient bacterial and *Synechococcus* abundances at station 7 (Wommack and Colwell, 2000).

Ambient *Synechococcus* populations were enumerated by filtering an appropriate sample volume onto a 25 mm 0.22 µm pore size black polycarbonate filter (Poretics Corp., Livermore, CA, USA), followed by enumeration with an epifluorescence microscope utilizing their natural autofluorescence under blue excitation (Vernet *et al.*, 1990). All slides were prepared immediately after sampling, refrigerated and counted within 24–48 h.

The most probable number (MPN) method was utilized to differentiate the subset of infectious *Synechococcus* cyanophages from the total viral population. All plates for enumeration of infectious cyanophage were prepared immediately after the sampling or experimental measurement. The sample to be enumerated was 0.2 µm filtered to remove any remaining host cells and a one to five dilution series of the sample was prepared using 96-well microtiter plates (Costar, Corning Inc., Corning, NY, USA). A susceptible *Synechococcus* host was then freshly 1:10 diluted and placed in each well (*Synechococcus* isolate WH7803). Control plates were prepared similarly using sterile SN media in the first column of wells. Three pseudoreplicate treatment and control plates were prepared from each sample. The plates were incubated in a light bank using cool white fluorescent light on a 14:10 light:dark cycle at room temperature until visibly dense growth of the host organism in negative controls was evident (10–14 days). Wells were scored as positive for virus if lysis of the host organism was apparent. Presence of viruses was confirmed in randomly selected lysed wells by SYBR Gold staining. Viral abundance was calculated for each plate using MPN method (Hurley and Roscoe, 1983). A most probable number calculator is available free of charge from the United States Environmental Protection Agency (<http://www.epa.gov>). All ambient and experimental samples were prepared in the same manner.

Total phytoplankton counts

Samples for phytoplankton cell counts were concentrated by reverse filtration (Dodson and Thomas, 1978) using 1 µm pore sized polycarbonate filters. Typically, 400 ml was concentrated to approximately 25 ml of which 5 ml was counted on a Zeiss inverted microscope following the procedures outlined by Hasle (Dodson and Thomas, 1978). The entire chamber was counted at $\times 100$ for larger species, while two to four chamber transects were counted at $\times 400$ for smaller species. Species identifications were based on descriptions in the

Tomas manual of phytoplankton identification (Tomas, 1997).

Viral reduction and prophage induction

Samples for prophage induction were pretreated by the viral reduction method to decrease the background level of viruses in the samples (Weinbauer and Suttle, 1996). Samples were pre-filtered through a 50 µm mesh to remove grazers and large phytoplankton, and then gently filtered through a 0.2 µm pore-size filter to a volume of approximately 5 ml to remove the majority of ambient viruses while retaining the bacterial population on the filter. Virus-free seawater (0.02 µm pore-size filtered) collected from the Gulf was added to the retentate to return it to the original sample volume. This procedure was repeated once, and then the filter was gently but thoroughly washed in the reconstituted sample to dislodge as many of the attached bacteria as possible before being discarded. The sample was then divided into control and treatment flasks. Treatment flasks were used for prophage induction experiments. A separate set of experiments was performed to evaluate the efficiency of recovery of ambient populations using this method. Our average recovery of ambient cells from this filter/vacuum-based method was 53.2% ($n=25$, data not shown).

Prophage inductions were incubated with Mitomycin C at a concentration of $1 \mu\text{g ml}^{-1}$. All samples were incubated in polymethylpropylene flasks and kept under 14:10 light:dark cycle at room temperature. Subsamples were taken from the treatment and control flasks at T_0 , T_4 , T_8 , and T_{24} . Slides were made immediately after sample collection for viral and bacterial enumeration as previously described. Slides were made in triplicate. The volume used for slides was dependent upon sample location and depth. Slides were foil wrapped and stored at -20°C until they could be counted; all slides were counted within a month after the completion of the cruise. Subsamples for *Synechococcus* and cyanophage enumeration were removed at T_0 and T_{24} , the slides and MPN plates were prepared immediately as for the ambient samples.

Primary and secondary productivity

Chlorophyll concentrations and primary productivity were measured at all stations and depths excluding stations 1 and $1 \times$. Primary productivity was measured in terms of the two photophysiological capacity parameters, maximal photosynthetic rate (P_{max}) and the photosynthetic yield or photosynthetic capacity ($P_{\text{max}}^{\text{B}}$). Samples taken in Niskin bottles were immediately transferred to 1 l light-shielded, acid-washed polyethylene bottles, of which 650-ml subsamples were spiked with 0.108 mCi of [^{14}C]bicarbonate (Amersham Pharmacia Biotech, Amersham Biosciences UK Limited,

<http://www.radiochemicals.amershambiosciences.com>) for a 0.167 μCi initial activity. Aliquots (40 ml) of the spiked water were transferred to 40-ml borosilicate vials and incubated in a photosynthetron apparatus (CHPT Mfg Co.) at *in situ* temperature and at irradiances ranging from 0 (dark sample) to $1000 \mu\text{E m}^{-2} \text{s}^{-1}$. Time-zero sample blanks were immediately filtered prior to commencement of incubation. Following incubation (1–2 h), samples were sequentially filtered onto 20, 2 and $0.2 \mu\text{m}$ 25-cm membrane filters and treated with $250 \mu\text{l}$ 10% HCl to allow unfixed [^{14}C]bicarbonate to evaporate. After 24 h, 10 ml of scintillation fluid was added, and sample radioactivity was determined by liquid scintillation counting in the channels ratio mode. The resulting data were plotted in P (productivity) versus E (irradiance) curves, and the photosynthetic parameters α (response to low light), $P_{\text{max}}^{\text{B}}$ (photosynthetic capacity normalized to chlorophyll a content), and β (susceptibility to photoinhibition) were computed using the exponential formulation of Platt *et al.* (1990).

Bacterial productivity was estimated by the leucine incorporation method as described by Kirchman *et al.* (1985). Ambient water samples from each station were split into two, a treatment and control. The control flask contained 5% trichloroacetic acid to kill the bacteria. 4,5- ^3H -leucine was added to both flasks to a final concentration of 8 nM. Three 10 ml subsamples were taken from each flask at T_0 and immediately filtered onto a $0.2 \mu\text{m}$ Millipore GS filter. Each filter was rinsed twice with 5% trichloroacetic acid followed by two rinses with 80% ethanol. Filters were dried in glass scintillation vials with foil-lined caps.

Treatment and control flasks were wrapped in aluminum foil and incubated at ambient water temperature in a deck-top incubator. Subsamples were taken at 60 and 120 min after T_0 and were filtered as described previously. Once the filters were dry, ethyl acetate was added to each vial to dissolve them. Ecoscint A was then added to each vial and the vials were allowed to sit for 24 h to ensure maximal dispersal into the counting fluid. Each vial was counted in a scintillation counter with read times of 30 s. Leucine uptake measurements were \log_{10} transformed before being used in statistical analysis.

Frequency of visibly infected cells

In addition to measuring prophage induction rates by the viral reduction technique, the frequency of visibly infected cells (FVIC) was measured at stations 1A, 1B, 1XS, 1XD, 2A, 2F, 4A, 7F, 7G, 8A and 8B (prophage induction stations). At each station, 10 ml of an ambient sample at T_0 was fixed with 2% final concentration of $0.2 \mu\text{m}$ filtered alkaline buffered formalin, in duplicate, and stored in the dark at 4°C until processed.

To prepare the samples for transmission electron microscopy, samples were ultracentrifuged at 20 000 r.p.m. onto a formvar-coated grid for 20 min. Grids were negatively stained for 30 s with uranyl acetate then rinsed three times with $0.02 \mu\text{m}$ filtered deionized water and air dried. The grids were then examined using a Hitachi 7100 electron microscope utilizing 125 KeV accelerating voltage to allow visualization of viral particles within the intact bacterial cells. Cells containing at least five phages were considered infected, and the total number of viral particles per cell was enumerated for an estimation of viral burst size (Noble, 2001). The FVIC was the proportion of total cells examined that contained visible viral particles. Burst size was the average number of viral particles observed within cells for each station. The frequency of infected cells (FIC) was estimated by the formula $\text{FIC} = 9.524 \text{FVIC} - 3.256$ (Weinbauer *et al.*, 2003).

The lysogenic fraction was calculated by the following equation:

$$\frac{[(V_i - V_c)/B_z]}{\text{Bact}_c} 100$$

In this equation, V_i is the number of viruses counted in the induced sample, V_c is the number of viruses counted in the control sample, B_z is the calculated burst size at T_0 estimated directly from the FVIC samples, and Bact_c is the number of bacterial cells counted in the control sample (Paul, 2001).

Statistical analyses

Two types of statistical analysis were performed. The first type was standard univariate statistical analyses with SigmaStat v. 3.5 and Minitab v. 13. The second type was multivariate statistical analyses performed using Primer v.5.2.9 software (Primer-E Ltd, Plymouth Marine Laboratory, UK www.primer-e.com).

All correlations were performed using the Pearson Product Moment Correlation in Minitab v. 13. To test the significance of the linear regression for lysogenic fraction versus leucine uptake, an analysis of variance was performed in SigmaStat using the linear regression option. One-way analysis of variance using the Holm-Sidak method as the *post hoc* test, a pair-wise multiple comparison procedure, was performed to determine which stations were significantly different from one another for the ambient viral and bacterial measurements. Viral and bacterial counts from the diel failed a normality test and so the Kruskal-Wallis one-way analysis of variance on ranks was used for this data. The *post hoc* test used was a Tukey multiple pair-wise comparison test. Principal component analysis of the phytoplankton data was performed using Minitab v. 13.

Prophage induction was defined as the difference between viral abundance in the Mitomycin C and the control treatments at T_{24} for all experiments (Weinbauer and Suttle, 1996). Viral counts from the

control and Mitomycin C treatment samples were compared by a Student's *t*-test to determine if prophage induction was significant at a given time point.

Multivariate analyses were also performed between cyanophage and heterotrophic induction parameters and measured environmental parameters. Initially, the similarity matrices of both the cyanophage induction parameters and environmental parameters that were constructed using normalized Euclidian distances were compared to determine whether a statistically significant relationship existed between the matrices utilizing the RELATE test (sample statistic ρ). The matrices were then compared by the BIOENV test using both untransformed and log-transformed data to determine which environmental parameters best accounted for the observed distribution of cyanophage or heterotrophic prophage induction.

Results

The distinct differences between the non-plume and plume stations in terms of salinity and microbial parameters can be seen in Figure 1 and Table 1. The non-plume stations were associated with higher salinity (range 34.2–35.9) and lower ambient levels

of all other parameters. At plume stations, total bacteria in the surface samples ranged from 1.41×10^6 to $4.34 \times 10^6 \text{ ml}^{-1}$. The span in non-plume stations was lower ranging from 5.98×10^5 to $9.46 \times 10^5 \text{ ml}^{-1}$ with viral abundance showing a similar pattern.

The depth profiles showed viral abundance was generally higher at comparable depths within plume station 3 than the more oligotrophic station 2. However, both stations had decreasing viral abundance with depth. By 100 meters, viral and bacterial populations at both stations were similar. As might be expected, the abundance of both *Synechococcus* and cyanophage decreased with increasing depth (Table 1). The non-plume station 2 was characterized by generally normal marine salinity, log-linear penetration of PAR, a large, distinct bimodal SCM at 40 and 88 m (Figure 1) and relatively low microbial abundances and activity (Table 1). Conversely, plume station 3 was characterized by a lens of low salinity, high turbidity surface water, high microbial abundances, a smaller SCM at 8 m, high viral abundances and high productivity (Figure 1; Table 1).

Synechococcus and infectious cyanophage abundances were also typically higher at plume stations. Interestingly, the infectious *Synechococcus* cyanophage abundance was significantly greater in the surface waters at plume station 3 (5.6×10^4

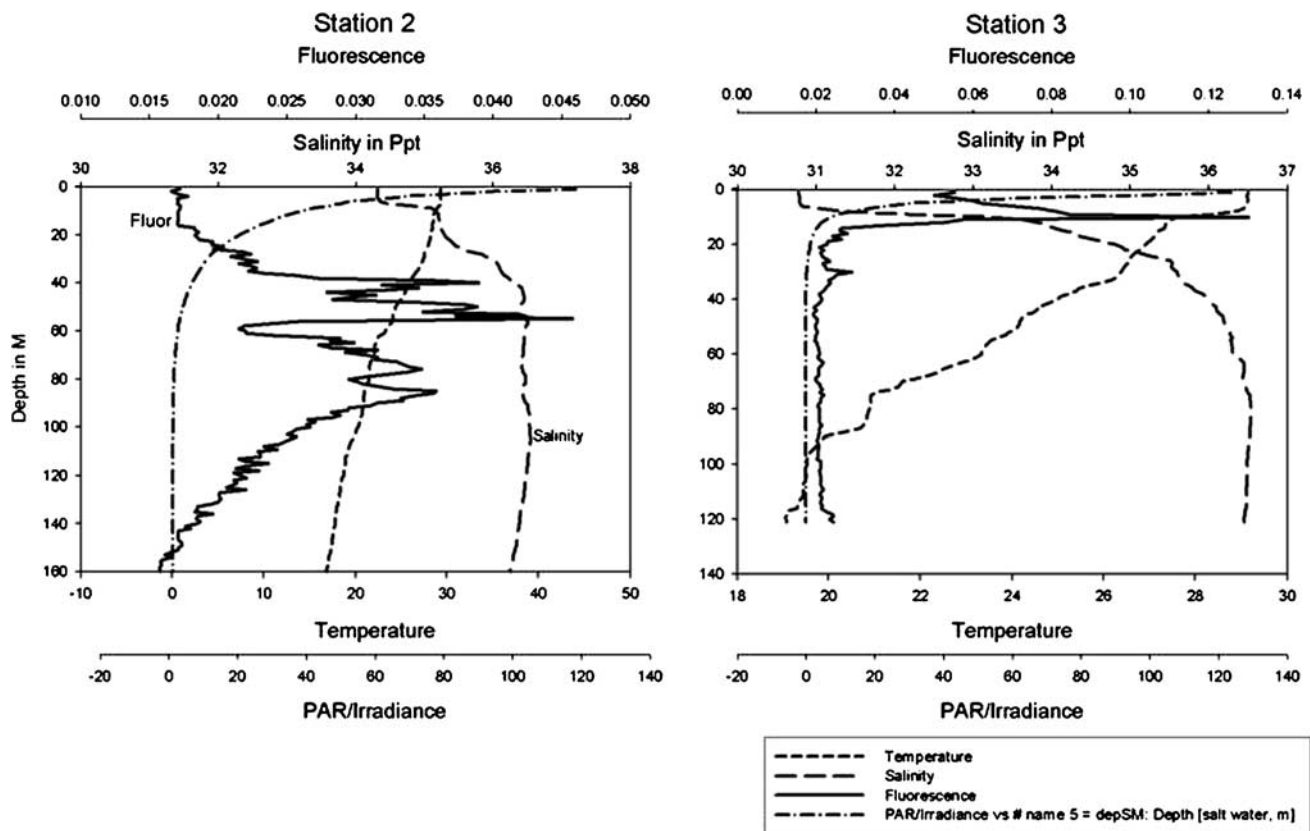


Figure 1 Comparative CTD depth profiles of an oligotrophic and plume station. Temperature, photosynthetically active radiation (PAR) salinity, and fluorescence (as a proxy for chlorophyll) are plotted versus depth in meters for the profiles performed at an oligotrophic station (station 2-left panel) and a hypertrophic plume station (station 3-right panel).

viruses ml^{-1}) than at the surface of non-plume station 2 (2.5×10^4 viruses ml^{-1}), although the *Synechococcus* cell abundances were not significantly different at these two stations (3.01×10^4 and 3.96×10^4 cells ml^{-1} , respectively). This indicates that other phytoplankton types were a dominant source of the high chlorophyll signature observed at station 3 (Figure 1).

In addition to measurements of the contrasting trophic environments, a diel study was performed in the distal plume to obtain an indication of the dynamics of heterotrophic and autotrophic microbial populations throughout a 24-h cycle (Figure 2). The average bacterial abundance over the course

of the diel was 2.96×10^6 cells ml^{-1} . Total heterotrophic viral abundance ranged from a maximum of 3.53×10^7 VLP ml^{-1} at 18:41 to a minimum of 1.61×10^7 VLP ml^{-1} at 10:25. However, there were no significant differences in total viral abundance over the course of the diel. Bacterial and viral abundances were highly correlated ($r=0.874$, $P<0.001$), indicating that, as bacterial populations increased or decreased, so did viral populations over the course of 24 h. The VBR for this time period ranged from 6.97 at 20:30 (station 7B) to 11.43 at 10:25 (station 7E).

The observed patterns of interaction between *Synechococcus* and its associated cyanophages were

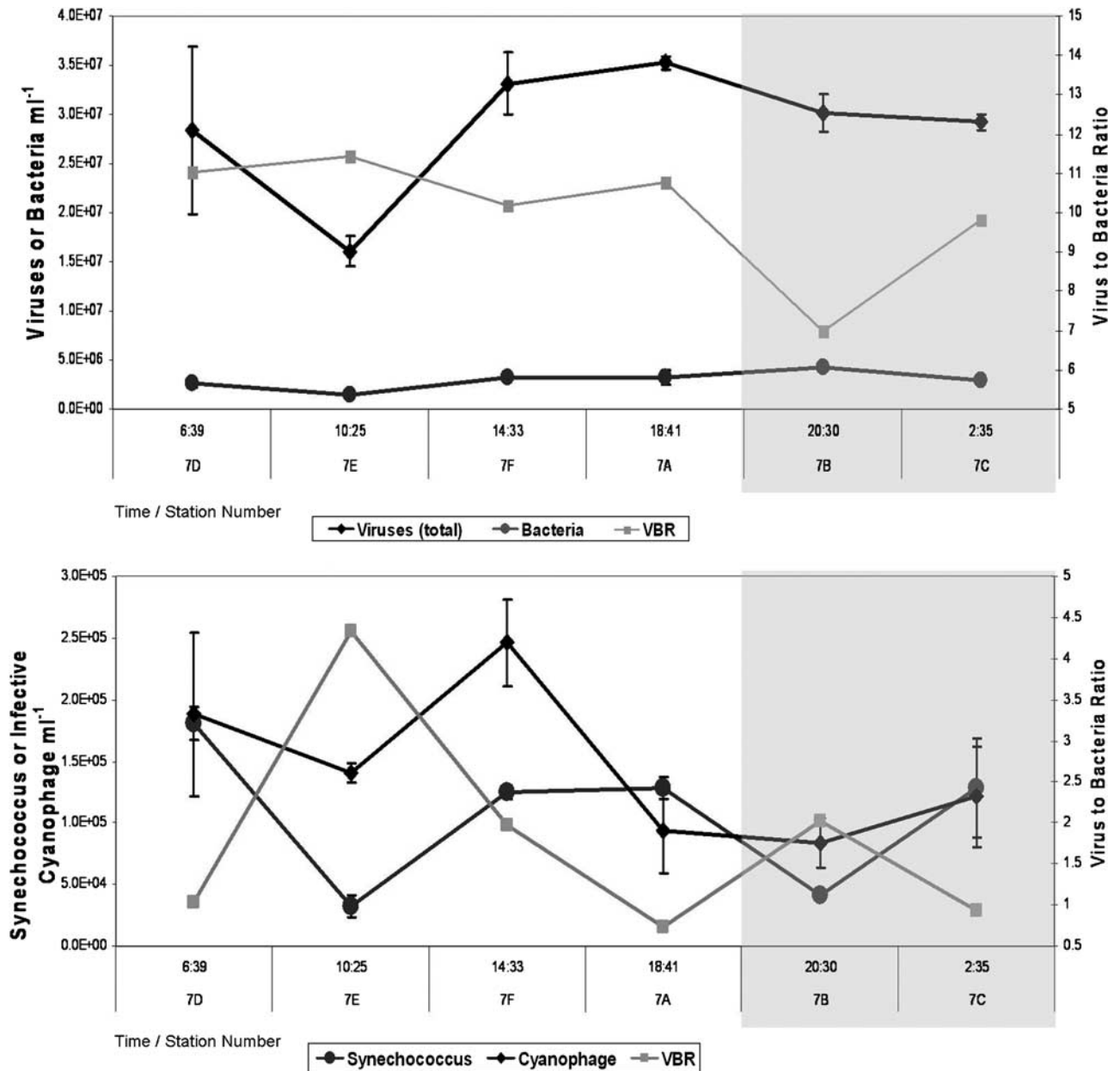


Figure 2 Diel study of microbial and viral abundances. The top panel depicts the total (that is heterotrophic) bacterial and viral abundances, and virus to bacteria ratio (VBR). The bottom panel shows the *Synechococcus* and infectious cyanophage abundances and VBR. Night-time samplings are shaded gray.

markedly different from the heterotrophic populations. Analysis of variance indicated that *Synechococcus* and infectious cyanophage abundances were significantly different between time points over the course of the diel ($P > 0.001$). *Synechococcus* abundance was highest at 1.81×10^5 cells ml⁻¹ at 06:39, shortly after sunrise, but the lowest cell abundance, 3.26×10^4 cells ml⁻¹, was observed at the next sampling point (Figure 2). Infectious cyanophage abundance and *Synechococcus* abundance were not as closely coupled as for the heterotrophic bacteria. The peak in host cell abundance was at 06:39 after which host numbers decreased but viral numbers increased. In contrast to the total (that is, heterotrophic) viral numbers, infectious cyanophage abundances were generally higher during the day and lower at night. The ratio of infectious cyanophages to *Synechococcus* cells ranged from 0.73 at 18:41 to 4.3 at 10:25. However, these observed patterns may reflect the measurement of the subset of infectious cyanophages.

Prophage induction experiments were performed using samples from both plume and non-plume environments, both at the surface and at the SCM. A total of 11 prophage induction experiments were carried out for the heterotrophic bacterial populations, and 9 were conducted for *Synechococcus* populations (Figure 3). No significant prophage induction events were observed for heterotrophic bacteria in any of the surface samples. However, three of the subsurface stations (1XD, 2F and 8B) had significant prophage induction at T₂₄. For the *Synechococcus* populations, significant induction of infectious cyanophage was detected in seven of

nine experiments. The only stations where *Synechococcus* cyanophage induction was not detected were highly productive surface plume stations.

For heterotrophic populations, the lysogenic fraction (%) was calculated for each station where heterotrophic induction experiments were conducted from T₀ burst sizes measured using the FIC method developed by Weinbauer *et al.* (2003). In this study, the minimum burst sizes in the MRP were estimated to be 21.7 viruses per cell, which was a known underestimate, as there were bacterial cells with viral particles that were too numerous to be counted in the samples from plume station 4A. In the oligotrophic waters of the Gulf, the average burst size was 18.92, which is similar to the reported average burst size of 19.8 for other oligotrophic environments (Parada *et al.*, 2006). As there was no statistically significant difference between the burst sizes obtained from the different stations, the combined average burst size of 18.72 viruses per cell was used for all calculations of lysogenic fraction. This value is well within the ranges previously reported for environmental marine lysogens and much lower than the frequently utilized burst size of 50. In surface samples, the value was typically negative and ranged from -0.03% to -2.01% (Table 1). Samples collected at 1XD, 2F, 7G and 8B, all of which were from the SCM or deeper, had positive lysogenic fraction values ranging from 1.67% to 6.94%. Stations 1XD, 2F and 8B were the stations where significant prophage induction was seen at T₂₄. The only SCM sample that did not have a positive lysogenic fraction was station 1B, a shelf station.

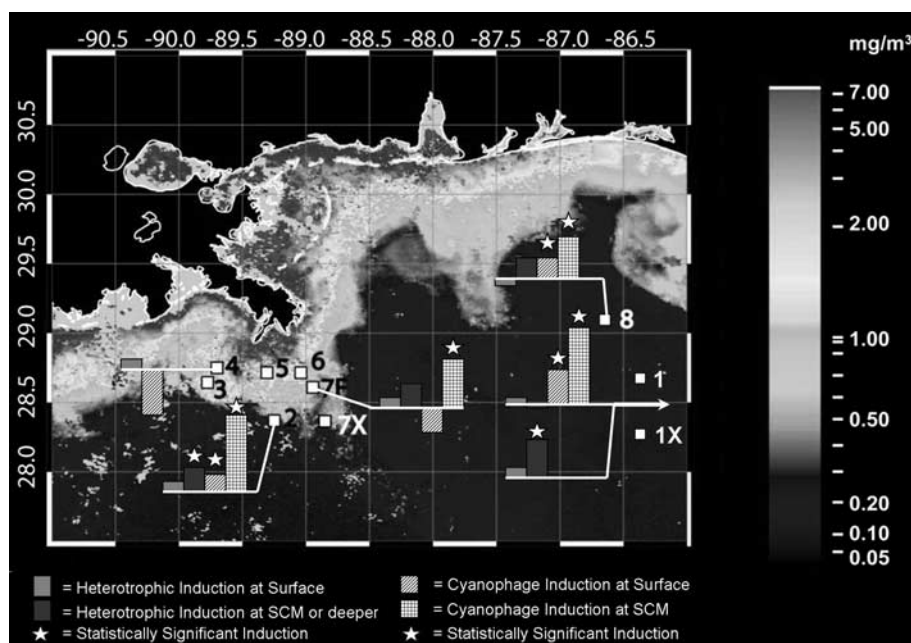


Figure 3 MODIS Satellite image of the Gulf of Mexico showing averaged surface chlorophyll with the cruise stations indicated. The arrow for station 1 and 1X indicates that these stations were east of the map boundary in the oligotrophic Gulf. The bars depict the relative level of prophage induction detected at each station where experiments were performed. Stars indicate a statistically significant level of prophage induction in that sample.

Linear regression and Pearson correlations were calculated between the lysogenic fraction and the log-transformed values of leucine uptake (Figure 4). The correlation coefficient between lysogenic fraction and leucine uptake was -0.876 ($P=0.02$), indicating a strong inverse relationship between the two parameters. Bacterial activity was also negatively correlated with depth, ($r=-0.815$, $P=0.002$), indicating that as depth increases, bacterial activity decreases.

The lysogenic fraction for *Synechococcus* populations cannot be measured by currently available culture-based methods. It is important to recognize that the MPN method measures only the subset of cyanophages infectious for the host used for enumeration. Due to this limitation, an accurate estimation of burst size cannot be calculated and VBR estimations are typically much lower than for

the heterotrophic (that is, total) bacteria. Nevertheless, despite being a subset of the total, the MPN method has demonstrated a similar level of precision to other methods of viral enumeration and is very useful for following trends in the population (Cottrell and Suttle, 1995). Molecular methods of enumeration of the subset of cyanophage have also been utilized (Fuller *et al.*, 1999); however, this method also has limitations in the ability to detect all cyanophages (McDaniel *et al.*, 2006).

For this analysis, the level of cyanophage induction, measured as the difference between the treatment and control abundances of infectious cyanophage, was normalized to the ambient level of *Synechococcus*. This value was compared to the \log_{10} -transformed values for primary productivity (P_{\max}). The same strong inverse relationship between lysogeny and productivity emerges ($r=-0.815$,

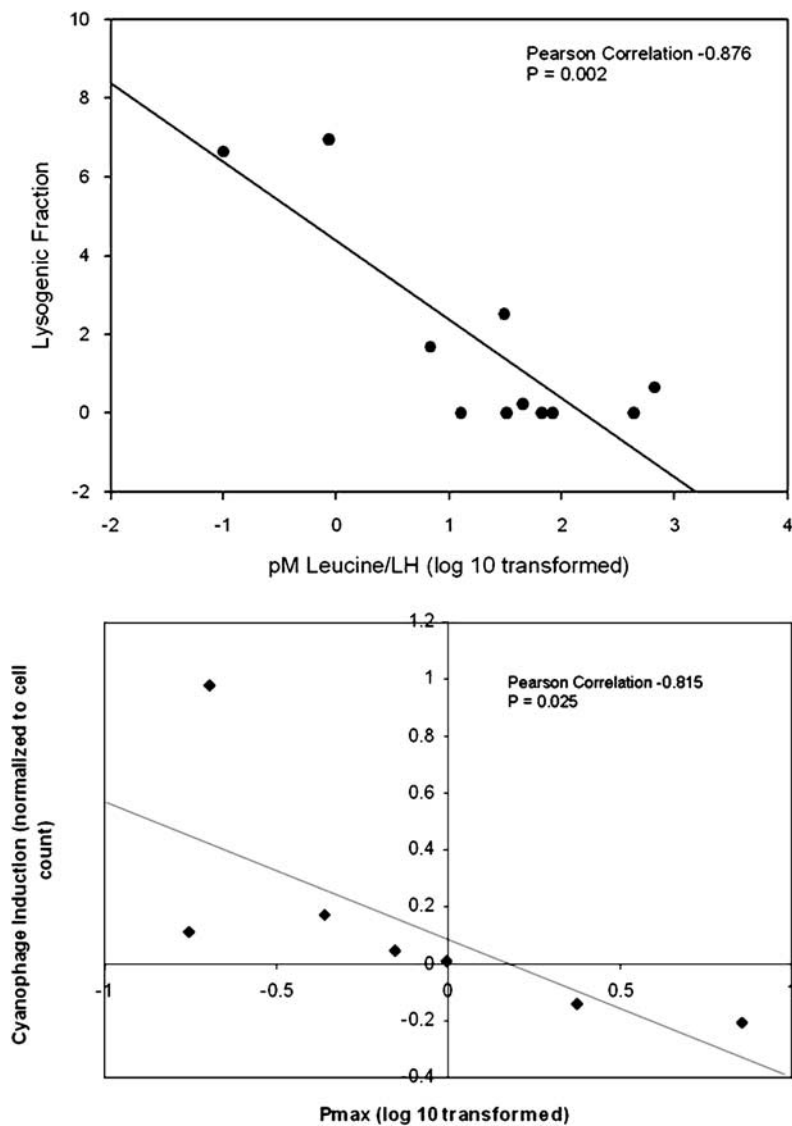


Figure 4 Comparison of lysogeny and productivity. The top panel is the linear regression of the heterotrophic lysogenic fraction versus bacterial activity (\log_{10} transformed). The bottom panel compares the level of cyanophage induction normalized to ambient *Synechococcus* abundance to the \log_{10} -transformed net primary productivity (P_{\max}).

$P=0.025$), analogous to that observed for heterotrophic bacterial populations (Figure 4).

Discussion

The ambient parameters illustrate the temporally and spatially similar environments with strongly contrasting trophic settings, which provide the framework for the main focus of these experiments, the investigation of patterns of lysogeny. In these experiments, a strong inverse relationship was observed between overall system productivity and lysogeny in both heterotrophic and autotrophic bacterial populations. Despite the similar relationship to productivity, clear differences were observed in the prevalence of lysogeny between heterotrophic bacteria and *Synechococcus* populations in the same samples. One of the primary differences between the two ambient populations was the relationship between cell abundance and salinity, with the heterotrophic bacteria demonstrating a close inverse relationship to salinity, where *Synechococcus* did not.

The measured surface ambient parameters as well as the depth profiles illustrated that the contrasting environments sampled had very different physical and microbial parameters (Figure 1; Table 1). The non-plume stations were characterized generally by normal marine salinity, log-linear penetration of PAR, a distinct SCM and relatively low microbial abundances and activity. Conversely, the plume stations were characterized by a lens of low salinity, high turbidity surface water, high microbial abundances, a smaller SCM, high viral abundances and high productivity. Disparate phytoplankton assemblages were also observed. The oligotrophic non-plume stations were dominated by populations of coccolithophores and cyanobacteria, while the plume stations were dominated by diatoms and dinoflagellates. In addition, microflagellates and ciliates were present in all samples with the largest populations observed in the plume samples.

The diel study demonstrated differing temporal patterns of phage–host interactions for heterotrophic bacteria and *Synechococcus*. The heterotrophic bacterial abundance and the total viral abundance closely covaried, leading to a generally stable VBR throughout the 24-h experiment with highest viral abundances at night. A high VBR is associated with a high level of bacterial productivity and viral lytic activity, this was consistent with the highest VBR observed at the 20:30 sampling (Wommack and Colwell, 2000). In contrast, the *Synechococcus* and their infectious cyanophage abundances were significantly variable and not as closely coupled, with highest viral abundances and VBR observed during the day.

As photosynthetic autotrophs, natural populations of *Prochlorococcus* and *Synechococcus* have been documented to follow diel patterns of growth

(Jacquet *et al.*, 2002). The recent discovery that many lytic cyanophages carry host-like photosynthesis genes indicates that the lytic cycle of their associated cyanophages is also light dependent (Lindell *et al.*, 2005; Sullivan *et al.*, 2006). Additionally, it has been verified for cyanophages S-PM2, AS-1 and AS-1M that phage adsorption is light dependent (Sherman, 1976; Jia and Mann, 2005; Kao *et al.*, 2005). It has been hypothesized that a wave of phage adsorption following dawn could lead to a release of progeny phage later in the day (Clokie and Mann, 2006). However, because of the long latent period of cyanophage it is likely the pattern observed in these experiments was the completion of a lytic infection begun the previous day (Clokie *et al.*, 2006b). A recent study of diel variations of infectious cyanophage in September in the Indian Ocean demonstrated a different temporal pattern over a similar short timescale, suggesting this pattern may vary by location or time (of year).

For the measurements of prophage induction in the heterotrophic bacterial populations, lysogeny appeared to be associated only with the SCM and deep subsurface stations in the oligotrophic Gulf of Mexico where bacterial populations were observed to be as low as 10^4 cells ml⁻¹ (Table 1). In the calculation of the lysogenic fraction, T_0 samples were used for the estimations of burst size. Using this method was expected to produce a more accurate estimate of lysogenic fraction, because burst size was measured directly in the experimental samples by transmission electron microscope rather than using the cited literature standard burst size of 50, which was derived from experiments in different environments than the ones we observed on this cruise (Hennes and Suttle, 1995). This made the assumption that the T_0 burst size would remain relatively consistent throughout the induction.

Previous studies have observed that lytic burst sizes and FIC increase towards eutrophic conditions (Weinbauer *et al.*, 2003; Bongiorni *et al.*, 2005). The mean value of burst sizes for different eutrophic environments is 24.9 viruses per cell (Parada *et al.*, 2006), but these values may not be directly applicable to temperate viruses. The burst size range for marine environmental lysogens has not been extensively studied; however, ranges from 11 to 45 viruses per cell have been reported (reviewed by Wommack and Colwell, 2000). Although the value used in this study is consistent with previous estimates of burst size from lysogens from in natural marine environments, there is potential for error in the measurement from using only the T_0 value. Nevertheless, the sign of the value indicating presence or absence of prophage induction and the basic trends would remain the same.

The heterotrophic lysogenic fraction measured in surface samples was typically negative (Table 1). In contrast, samples from the SCM or deeper had positive lysogenic fractions associated with proph-

age induction. The only SCM sample that did not have a positive lysogenic fraction was station 1B, a shelf station. Negative lysogenic fraction values occur when viral abundance in the control treatment is greater than viral abundance in the Mitomycin C treatment. This has been observed in non-inducing microbial populations and is most likely due to the extreme toxicity of Mitomycin C.

It should be mentioned here that although Mitomycin C has been widely used in prophage induction experiments, it is an extremely toxic anti-neoplastic agent (Windholz *et al.*, 1976). Mitomycin C acts as an alkylating agent and causes prophage induction by direct DNA damage (Iyer and Syzbalski, 1963) and activation of the *recA* repair system. As a result of this toxicity, a decrease in viral production is often observed in treated samples when prophage induction is not present.

For heterotrophic bacterial populations, multivariate statistical analysis indicated that the ambient level of viral abundance and productivity was inversely related to prophage induction and both factors combined are most predictive of lysogeny ($\rho = 0.899$, $P = 0.001$). Standard statistical methods also demonstrated a strong inverse relationship between lysogenic fraction and depth as well as bacterial productivity and depth. Therefore, as bacterial activity decreases with depth, the lysogenic fraction increases, providing an explanation for the presence of heterotrophic prophage induction in subsurface samples only.

In contrast to the heterotrophic bacteria, infectious cyanophage prophage induction was much more prevalent in the *Synechococcus* populations. In this case, statistically significant cyanophage induction was detected in 7 of 9 experiments performed (77.8%) encompassing all oligotrophic surface stations as well as all subsurface stations measured (Figure 3). Only in the highly productive plume surface stations was cyanophage induction not detectable. There was a similar inverse relationship between *Synechococcus* cyanophage induction and primary productivity. Nevertheless, it appeared that the *Synechococcus* populations were less likely than the heterotrophic bacteria to 'switch' from lysogenic to lytic viral production.

For *Synechococcus*, multivariate statistical analysis indicated that a low level of ambient cyanophage abundance was the single factor most predictive of lysogeny ($\rho = 0.862$, $P = 0.005$). This supports previous observations, at least for ambient *Synechococcus* populations (McDaniel and Paul, 2005). However, conventional statistical analyses also indicated a strong inverse relationship between lysogeny in *Synechococcus* and the log-transformed primary productivity measurements.

Since heterotrophic and autotrophic populations respond similarly to overall system productivity, what was it that accounted for the differences in the prevalence of lysogeny?

The heterotrophic bacterial abundance demonstrated a strong inverse relationship with salinity ($r = -0.931$, $P < 0.001$). Interestingly, leucine uptake was also at its highest levels at stations located within the MRP. The answer may lie in the more rapid growth and adaptability of heterotrophic bacteria in comparison to *Synechococcus* strains. It has been observed in the lab that cultures of heterotrophic bacteria grow much more vigorously than *Synechococcus* isolates. Unlike heterotrophic bacterial cultures that respond immediately to high levels of nutrients and reach log-phase of growth in several hours, while *Synechococcus* cultures can take several days (data not shown). The resident marine bacteria are likely to be able to respond more quickly to the high nutrient levels in the plume. In addition, the river plume carries its own microbial population, which may have a lower prevalence of lysogenic bacteria. The correlation between high abundances of bacteria and lower salinity in these samples may represent rapid growth of resident marine populations, shifts in community composition or a combination of both.

Synechococcus abundances were not highly correlated or significantly inversely related to salinity. Although overall primary productivity is highest in plume samples, whole and 0.2 μm size-fractionated primary productivity demonstrated that a higher proportion of the total primary productivity in the plume was driven by larger phytoplankton, such as diatoms, rather than *Synechococcus* (John *et al.*, 2007).

These findings suggest that the heterotrophic bacterial populations were more adaptable than *Synechococcus* populations to the low salinity, high nutrient environment of the MRP, which may be due to high growth rates in response to rapid nutrient inputs, shifts in the community composition, or both. Since lysogeny is presumed to be an adaptation to adverse conditions this plasticity of community composition and/or metabolism was a likely contributor to the lower prevalence of prophage induction in the heterotrophic populations in the vicinity of the plume.

In conclusion, these experiments failed to support both null hypotheses. Firstly, a strong inverse relationship was observed between overall system productivity and lysogeny in both heterotrophic and autotrophic bacterial populations. Second, clear differences were observed in the prevalence of lysogeny between heterotrophic bacteria and *Synechococcus* populations in the same samples. It was observed that one of the primary differences between the two ambient populations was the relationship between abundance and salinity, with the heterotrophic bacteria demonstrating a close inverse relationship to salinity, where *Synechococcus* did not. The low prevalence of lysogeny in the more diverse heterotrophic bacterial population may be due to greater adaptability of the community

to the lower salinity and high nutrient conditions in the vicinity of the MRP.

Acknowledgements

Funding for this project was provided by the National Science Foundation Biocomplexity program, grant number OCE-0221763. Thanks to the following people for their assistance: the Captain and crew of the UNOLS vessel 'Pelican' for making this study possible; Dr Jorge Corredor at the University of Puerto Rico for providing the primary productivity measurements; and Dr Gabriel Vargo at the University of South Florida, College of Marine Science for performing the phytoplankton counts.

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