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Viral lysis and nanoflagellate grazing as factors controlling diel variations of *Synechococcus* spp. summer abundance in coastal waters of Taiwan

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ABSTRACT: Diel variations in the protozoan grazing and viral-mediated mortality of *Synechococcus* spp. were simultaneously estimated using a modified dilution method in the coastal waters off Taiwan during 4 cruises in summer 2011. During the study period, the abundance of *Synechococcus* spp. showed a clear diel cycle and ranged from 1.2×10^4 cells ml^{-1} at 08:00 h to 9.8×10^4 cells ml^{-1} at midnight. The frequency of dividing cell (FDC) values were highest between afternoon and dusk and decreased to <10% between 20:00 and 08:00 h. *Synechococcus* spp. specific growth rates varied during the experimental period from 0.025 to 0.033 h^{-1} and 0.050 to 0.085 h^{-1} at daytime and nighttime, respectively. Rates of *Synechococcus* spp. mortality due to nanoflagellate grazing averaged 0.031 h^{-1} during daytime and 0.041 h^{-1} at night. The estimated mortality due to viral lysis was between 0.011 and 0.019 h^{-1} during daytime and between 0.026 and 0.065 h^{-1} at night. Nanoflagellate grazing was the dominant cause of *Synechococcus* spp. mortality during daytime; however, mortality from viral lysis and nanoflagellate grazing was more balanced at night.

KEY WORDS: *Synechococcus* spp. · Modified dilution method · Viruses · Nanoflagellates

INTRODUCTION

The fate of primary production has important implications for food web structure and biogeochemical cycling. In oceanic communities, picoplankton often dominate phytoplankton biomass and productivity (Waterbury et al. 1986, Olson et al. 1990, Blanchot & Rodier 1996, Agawin et al. 2000) and thus play a key role in marine carbon and nutrient cycles (Campbell et al. 1994). These picophytoplankton cells are not likely to sink due to their small size (Raven 1998), and grazing by nanoflagellates is frequently considered the major cause of mortality (Sanders et al. 2000, Sherr & Sherr 2002, Kimmance et al. 2007, Tsai et al. 2008, 2009). However, phytoplankton mortality from lysis has been described (Agusti et al. 1998, Alonso-Laita & Agusti 2006), and viruses may also control picophytoplankton abundance through high lytic activities (Baudoux et al. 2006, 2008, Kimmance et al. 2007). The flow of matter and energy through the food web is different depending on whether cells are grazed or lysed (Fuhrman 1999). Grazed production is at least partly passed to higher trophic levels, whereas viral lysis recycles nutrients and carbon within the microbial loop (Gobler et al. 1997).

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Synechococcus spp. show distinct diel changes in abundance, with higher division rates at dusk (Dolan & Šimek 1999, Vaulot & Marie 1999, Christaki et al. 2002, Tsai et al. 2009) and maximum abundances at night (Christaki et al. 2002, Tsai et al. 2009). Christaki et al. (2002) reported that the ingestion rates of heterotrophic nanoflagellates (HNF) were highest at night and declined during the daytime. Furthermore, the highest ingestion rates by nanoflagellates occurred after *Synechococcus* spp. cell division (Dolan & Šimek 1999, Tsai et al. 2009). These results are consistent with the control of *Synechococcus* spp. via consumption by heterotrophic and/or pigmented nanoflagellates.

Another potential source of cyanobacterial mortality is lysis by viruses (Suttle 2000). Cyanophages that infect *Synechococcus* spp. are often abundant, with concentrations coupled to those of their putative hosts (Wang & Chen 2004), but viral lysis of *Synechococcus* spp. is not always observed or is sometimes low compared with viral lysis of eukaryotic phytoplankton (Baudoux et al. 2007, 2008). Suttle (2000) suggested that viral infection and lysis of *Synechococcus* spp. vary during the diel cycle, akin to the observed periodicity for protistan ingestion noted above. This could affect determinations of viral-induced mortality.

The interplay between viruses and nanoflagellates in the control of aquatic prokaryotes is still poorly understood (Pernthaler 2005, Miki & Jacquet 2008). Bacterivory by pigmented nanoflagellates (PNF) appear to be the underlying biological factor regulating diel variations in *Synechococcus* spp. in coastal water of the subtropical western Pacific during summer (Tsai et al. 2009). However, no data exist on the effect of viral-mediated mortality for *Synechococcus* spp. for this area. The goal of the present study is to simultaneously compare the roles of viral lysis and nanoflagellate bacterivory in the diel variability of *Synechococcus* spp. in these waters during summer.

MATERIALS AND METHODS

Sampling

Samples were collected once a month from May to August 2011 (30 May, 27 June, 21 July and 23 August) at an established coastal station (25° 09.4′ N, 121° 46.3′ E) along a rocky shore in northeastern Taiwan (Fig. 1). The environment of this site has already been described, based on data gathered from 1999 to 2001 (Tsai et al. 2005).

Samples for the enumeration of *Synechococcus* spp., heterotrophic nanoflagellates (HNF), pigmented nanoflagellates (PNF) and viruses were collected at 2 h intervals for 24 h (for 12 h in May). On each sampling day, seawater was collected twice for modified dilution experiments, from 08:00 to 09:00 h in the morning and 20:00 to 21:00 h in the evening (local time). The water temperature was measured immediately after the sampling bucket was cast, and all samples were brought to the laboratory within 30 min.

Dilution experiments

We estimated viral lysis and nanoflagellate grazing rates using a modified technique of parallel dilution experiments: a 'standard' set that reduces grazers and a set that reduces both grazers and viruses (Evans et al. 2003). Filter holders and incubation bottles were acid-cleaned with 10% HCl and rigorously rinsed with Milli-Q water. To prepare the 'standard' diluent, the natural sample was passed through 10 µm mesh and then filtered through 47 mm Nuclepore filters (type PC, pore size of 0.2 µm). A filtered seawater sample (<10 µm) was then diluted with the 0.2 µm filtered seawater in a 4-point dilution series consisting of 25, 50, 75 and 100% seawater (<10 µm). The series was incubated in 50 ml polycarbonate bottles for 8 h under continuous illumination (~150 µmol quanta m^{-2} s^{-1}) in thermo-controlled incubators (24°C in May and 28°C in the other months). The size fractionation used for grazers (<10 µm) was chosen based on previous studies at this site to eliminate ciliates but not nanoflagellates (Tsai et al. 2011). An

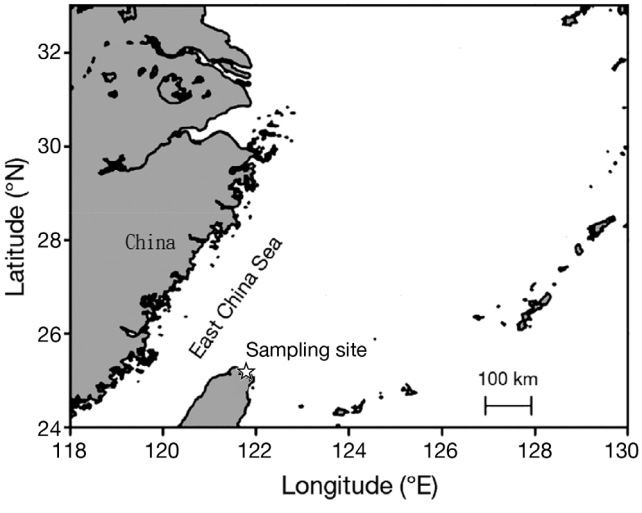

Fig. 1. Sampling site (☆) in coastal waters of the western subtropical Pacific

additional dilution series, using 10 kDa filtered seawater in place of 0.2 µm filtered water, modified both grazing and viral mortalities. The net growth rate of *Synechococcus* spp. (k, h^{-1}) was calculated for each sample based on microscopic cell counts at the start and the end of the experiment (N_{t0} and N_t) assuming exponential growth (Landry & Hassett 1982):

$$k = \ln(N_t / N_{t0}) / (t - t_0) \quad (1)$$

The regression coefficient of the apparent growth rate versus dilution factor for the 0.2 µm dilution series, though usually interpreted as protistan grazing (m_g) (Landry & Hassett 1982), actually includes viral mortality (m_v) because most viruses pass through a 0.2 µm pore (Evans et al. 2003). However, when virus-free seawater (10 kDa filters) is used as a diluent, the regression reflects release from both grazing and viral mortality ($m_g + m_v$), and a direct estimate of viral mortality for *Synechococcus* spp. can be obtained from the difference in the slopes of the regression lines between the 2 dilution series.

Viral, *Synechococcus* spp. and nanoflagellate abundance counts

Viruses, *Synechococcus* spp. and nanoflagellates were counted using an epifluorescence microscope (Nikon Optiphot-2) at 1000× magnification. Viruses were processed with a slight modification of a protocol described by Noble & Fuhrman (1998). Briefly, samples from 0.5 to 1 ml were filtered on Anodisc filters (0.02 µm pore size, Whatman) backed by 0.45 µm pore size Millipore filters. The samples were then placed on drops of SYBR Green I (Molecular Probes) solution diluted at 1:400 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stained for 15 min in the dark. The membranes were then placed on glass slides and treated with 25 µl of 50% glycerol and 50% PBS buffer (0.85% NaCl, 0.05 M NaH_2PO_4, pH 7.5) containing 0.1% *p*-phenylenediamine as antifade and mounting agents. Subsamples of 1 to 2 ml or 20 ml were filtered onto 0.2 µm or 0.8 µm black Nuclepore filters for *Synechococcus* spp. and nanoflagellates, respectively. Samples for counting nanoflagellates were stained with DAPI at a final concentration of 1 µg ml^{-1} (Porter & Feig 1980); PNF and HNF were distinguished based on the presence or absence of chlorophyll autofluorescence. *Synechococcus* spp. were identified by their orange autofluorescence under blue excitation light. Dividing cells of *Synechococcus* spp. were also enumerated, and the frequency of dividing cells (FDC) was examined. To obtain reliable estimates of abundance, we counted 30, 30 and 50 fields of view for viruses, *Synechococcus* spp. and nanoflagellates, respectively.

Statistical analysis

Least-square regression analysis was performed to determine the relationship between the *Synechococcus* spp. growth rate and fraction of 10 kDa or 0.2 µm dilution series. The significance of the regression lines was tested using an analysis of variance (ANOVA). The significance of differences between the slopes of the 10 kDa and 0.2 µm dilution series was determined using an *F*-test. When the slopes were significantly different, the magnitude of viral mortality was assessed. STATISTICA 7.0 software was used for all statistical operations.

RESULTS

Diel variations of *Synechococcus* spp., nanoflagellate and viral concentrations

The sea surface temperature at the coastal station was generally highest at 14:00 h and cooler at night for all sampling dates, though the temperature in May was cooler than that of the other months investigated (Fig. 2A). FDC values showed a strong diel variation pattern, with the greatest abundance of dividing cells present between afternoon and dusk and decreasing to <10% between 20:00 h and 08:00 h (Fig. 2B). The highest FDC was ~50% in the June and August samples compared to 30 to 35% in May and July (Fig. 2B). The greatest diel change in the abundance of *Synechococcus* spp. occurred in July when the population size increased from 1.2×10^4 cells ml^{-1} at 08:00 h to 9.8×10^4 cells ml^{-1} at midnight (Fig. 2C). During the other months, the diel change in *Synechococcus* spp. abundance had a reduced amplitude, with the highest values (5.8×10^4 cells ml^{-1}) recorded at 20:00 h and gradually decreasing through the night (Fig. 2C).

HNF and PNF abundance ranged from 0.45×10^3 to 1.51×10^3 cells ml^{-1} and 0.58×10^3 to 2.80×10^3 cells ml^{-1}, respectively (Fig. 3A,B). PNF showed clear diel patterns in abundance, with the highest values recorded at dusk (August) or night (June and July) during the study period (Fig. 3B). No diel rhythm was evident for either HNF or viral abundance (Fig. 3A,C).

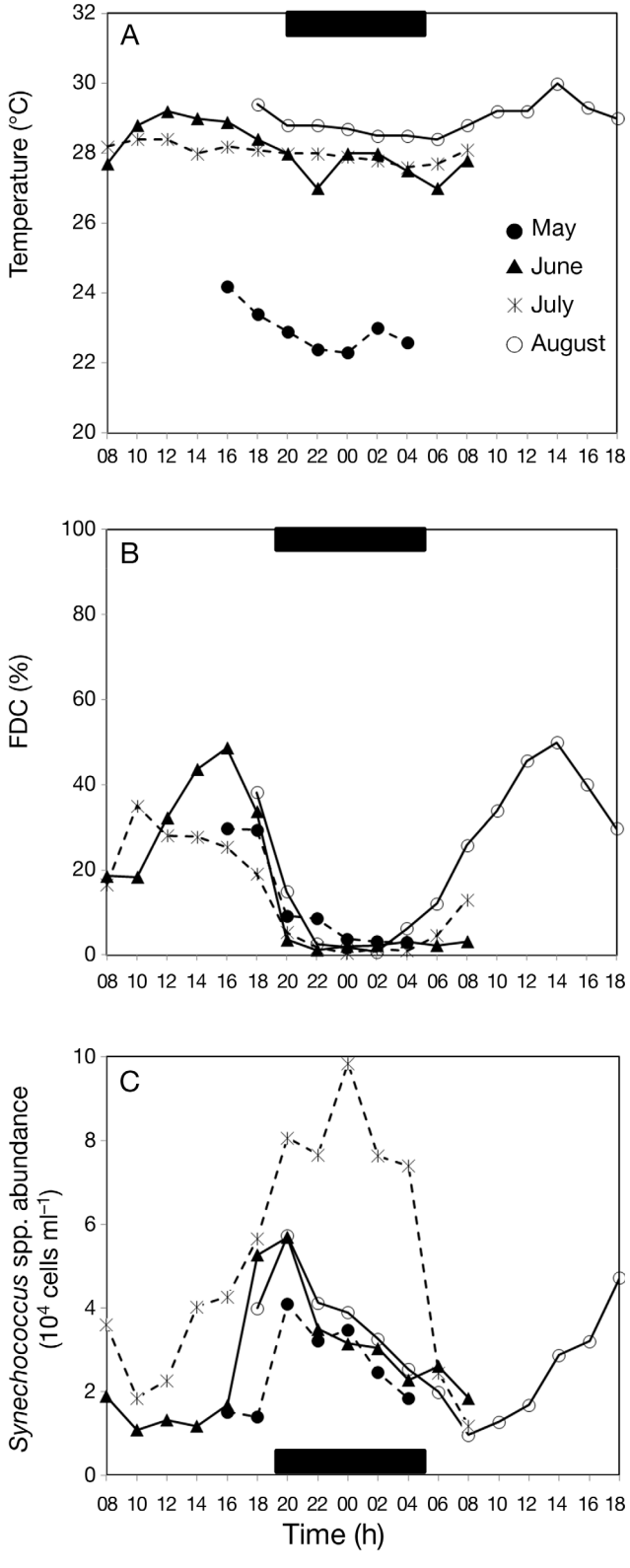

Fig. 2. Diel variations of (A) surface water temperature, (B) frequency of dividing cell (FDC) and (C) *Synechococcus* spp. abundance between May and August 2011. The black bar represents the dark period

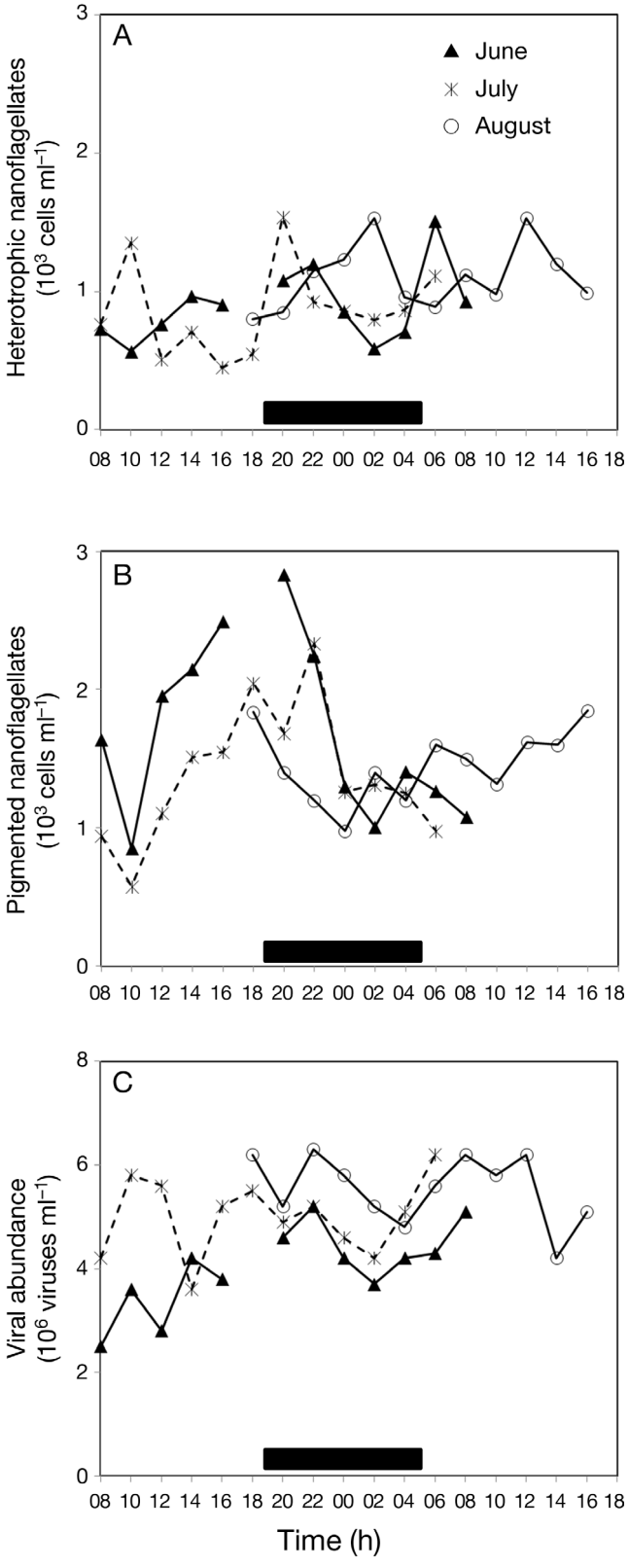

Fig. 3. Diel variations in (A) heterotrophic nanoflagellate, (B) pigmented nanoflagellate and (C) viral abundance between June and August 2011. Data not available for 18 June

Synechococcus spp. growth and mortality in dilution experiments

The *y*-intercepts of the regression lines (*Synechococcus* spp. specific growth rates) for the 0.2 µm and 10 kDa fractionated series were, on average, 0.006 and 0.029 h^{-1} for the daytime experiments and 0.011 and 0.063 h^{-1} for the nighttime experiments (Table 1). The regression coefficients (slopes), which represent *Synechococcus* spp. mortality, ranged from 0.025 to 0.034 h^{-1} and 0.044 to 0.052 h^{-1} for the 0.2 µm and 10 kDa fractionated series, respectively, in daytime experiments (Table 1); there were significant differences between the slopes for the different fractionations ($p < 0.05$). For nighttime experiments, the values of regression coefficients ranged from 0.035 to 0.047 h^{-1} and 0.061 to 0.105 h^{-1} for the 0.2 µm and 10 kDa fractionated series, respectively (Table 1). The differences between the slope values indicated that *Synechococcus* spp. mortality due to viral lysis (m_v) ranged from 0.011 to 0.019 h^{-1} and 0.026 to 0.065 h^{-1} for daytime and nighttime experiments, respectively; the nighttime values were always higher than those for the daytime experiments (*t*-test, $p < 0.05$) (Table 1). Rates of *Synechococcus* spp. mortality due to nanoflagellate grazing (m_g) determined from the standard 0.2 µm fractionated series varied from 0.025 to 0.034 h^{-1} and 0.035 to 0.047 h^{-1} at daytime and nighttime, respectively (Table 1), and also showed higher values at night (*t*-test, $p < 0.05$). In the present study, nanoflagellate grazing was responsible for most of the *Synechococcus* spp. mortality (66% of the total) during daytime. However, mortality due to viral lysis increased in relative importance and was essentially equal to nanoflagellate grazing mortality at night (51.2% vs. 48.8% of the total mortality) (Table 1). Furthermore, grazing rate and viral lysis were positively correlated with *Synechococcus* spp. growth (grazing: $r = 0.66$, $p < 0.05$; viral lysis: $r = 0.97$, $p < 0.05$).

Table 1. Linear regression analysis of 0.2 µm and 10 kDa dilution series. Standard errors in parentheses. *Regressions with significant ($p < 0.05$) mortality slopes and differences between the slopes of the 0.2 µm and 10 kDa dilution series. Samples collected only at night in May

Period	Fraction	Growth rate µ (h^{-1})	Grazing rate m_g (h^{-1})	Lysis rate m_v (h^{-1})	Ratio of lysis (%) ($m_v/[m_g+m_v]$)
May					
Day	0.2 µm	—	—	—	—
	10 kDa	—	—	—	—
Night	0.2 µm	0.013* (0.004)	0.035 (0.008)		
	10 kDa	0.05* (0.003)		0.026* (0.006)	42.6
June					
Day	0.2 µm	0.009* (0.005)	0.034 (0.007)		
	10 kDa	0.025* (0.003)		0.018* (0.007)	34.6
Night	0.2 µm	0.001* (0.011)	0.04 (0.011)		
	10 kDa	0.085* (0.024)		0.065* (0.026)	61.9
July					
Day	0.2 µm	0.001* (0.006)	0.034 (0.003)		
	10 kDa	0.028* (0.008)		0.011* (0.006)	24.4
Night	0.2 µm	0.018* (0.006)	0.047 (0.005)		
	10 kDa	0.063* (0.009)		0.043* (0.008)	47.8
August					
Day	0.2 µm	0.009* (0.003)	0.025 (0.002)		
	10 kDa	0.033* (0.006)		0.019* (0.006)	43.2
Night	0.2 µm	0.012* (0.004)	0.041 (0.003)		
	10 kDa	0.055* (0.006)		0.038* (0.007)	48.1
Mean					
Day	0.2 µm	0.006 (0.006)	0.031 (0.005)		
	10 kDa	0.029 (0.004)		0.016 (0.004)	34 (9.4)
Night	0.2 µm	0.011 (0.007)	0.041 (0.005)		
	10 kDa	0.063 (0.015)		0.043 (0.016)	51.2 (8.3)

DISCUSSION

Several methods have been used to estimate viral mortality of components of the microbial food web. These methods include tracking changes in viral abundance, production or decay rate (Heldal & Bratbak 1991, Wilhelm et al. 2002) and measuring the frequency of virus-infected bacterial cells (Proctor et al. 1993, Binder 1999). These methods use several assumptions about latent periods and/or burst sizes for viruses, and one advantage of the modified dilution method used here is that it gives insight into the quantitative significance of both viral lysis and grazing without the need for conversion factors (Jacquet et al. 2005).

The use of parallel dilution series involving the reduction of both viruses and microzooplankton (Evans et al. 2003) was previously used to estimate the mortality of the haptophyte *Phaeocystis globosa* (Scherffel) in temperate coastal waters (Baudoux et al. 2006), bacterioplankton in a mesotrophic lake (Jacquet et al. 2005), picophytoplankton in the North Sea (Baudoux et al. 2008) and assorted microbial groups in an estuary of the Gulf of Mexico (Ortmann et al. 2011). A modifica-

tion of the approach identified significant viral-induced mortality in bacterial populations off coastal Japan (Taira, et al. 2009). However, studies examining the relative importance of virus- and grazing-induced mortality in photosynthetic prokaryotes are still relatively rare (Baudoux et al. 2007, 2008, Ortmann et al. 2011). To our knowledge, the present study is the first to assess these components of *Synechococcus* spp. mortality together in the subtropical Pacific Ocean. Our data suggest that nanoflagellate grazing was the dominant cause of *Synechococcus* spp. mortality during daytime in this area, but viral lysis also was notable, especially at nighttime. These results are in contrast to previous studies in oligotrophic environments that consistently reported low viral infection rates and/or mortality for *Synechococcus* spp. (Suttle & Chan 1994, Garza & Suttle 1998, Baudoux et al. 2007, Baudoux et al. 2008).

The original dilution protocol was designed to determine the grazing of phytoplankton, and nutrient amendment was suggested to ensure that phytoplankton growth rates were independent of the dilution effect (Landry & Hassett 1982). However, some more recent studies question the efficacy of this part of the procedure, especially for the modified method that also examines viral mortality. Worden & Binder (2003) reported that the addition of nutrients stimulated microzooplankton-induced mortality rates of cyanobacteria in oligotrophic environments, leading to an overestimation of microzooplankton grazing rates. This was probably due to improvement of food quality of the cyanobacteria cells. Addition of nutrients also was reported to lead to an increase in viral burst size, thereby increasing viral production (Weinbauer et al. 2003), which could lead to an overestimation of viral-induced mortality. Kimmance & Brussaard (2010) also recommend against nutrient addition in dilution experiments because of the potential to produce unnatural growth rates. For these reasons, nutrients were not added to the incubations in the present study.

A key factor for the utility of any of the dilution methods is the accuracy of the apparent prey/host growth rate measurements that determine the slope and intercept of the regression. Some previous studies suggested that dilution in virus-free water reduces the growth rates of picophytoplankton, including *Synechococcus* spp. (Suttle 2000, Kimmance et al. 2007). Explanations for these observations included the possibility that *Synechococcus* spp. growth was stimulated by enhanced recycling of nutrients due to viral lysis of hosts in seawater containing viruses (0.2 µm diluent) compared to the virus-free water (Kimmance et al. 2007). Weinbauer et al. (2011) also provided persuasive evidence that the presence of active viruses stimulated *Synechococcus* spp. growth. In our study, however, we observed higher *Synechococcus* spp. growth rates in the 10 kDa than in the 0.2 µm series (Table 1), as did Baudoux et al. (2007, 2008), which suggests that factors besides viral lysis were stimulating picophytoplankton growth rates. Nonetheless, if the relatively high viral mortality that we observed was stimulating growth in the 0.2 µm diluent series but not in the 10 kDa diluent series, then the maximum *Synechococcus* spp. growth may have been even greater than the regression predicted. This could partly explain why the combined grazing and viral mortality always exceeded *Synechococcus* spp. growth in our incubations (Table 1). Fluctuations in the balance of growth and mortality are common, but the consistently higher mortality that we observed in our study is obviously unsustainable in the long term. To compare the rate measurements to observed net changes in abundance, we calculated the net growth rate as $\mu_{net} = \mu - (m_g + m_v)$. For the 4 nighttime analyses (Table 1), the μ_{net} estimates were negative and correspond with the observed decrease in *Synechococcus* spp. abundance (Fig. 2C). However, the μ_{net} estimates were also negative for the daytime analyses, suggesting that the dilution method may underestimate growth or overestimate the grazing or lysis rate. A possible factor that may have contributed to an underestimation of *Synechococcus* spp. production is the temperature during daytime. Previous studies revealed temperature as an important parameter affecting growth of *Synechococcus* spp. (Chang et al. 1996, Tsai et al. 2005). In the June and August experiments, the thermo-controlled incubators were as much as 2°C cooler than the maximum sea surface temperature (Fig. 2A), which would have reduced *Synechococcus* spp. growth rates in those experiments. Furthermore, an experimental study by Pradeep Ram & Sime-Ngando (2010) inferred that lysogeny tends to occur during periods of poor host cell abundance. Thus, in the viral dilution study, it is likely that the estimate of lysis in the more diluted treatment (25 to 50% fraction seawater dilution) was affected and the *Synechococcus* spp. growth rates were underestimated in our incubated samples.

Synechococcus spp. often exhibit a clear diel pattern, with higher division rates at dusk resulting in maximum abundance at night (Dolan & Šimek 1999, Christaki et al. 2002, Tsai et al. 2005, 2009). Those investigations, including one at the same site as that sampled in our study (Tsai et al. 2009), suggested that

the diel fluctuations of *Synechococcus* spp. abundance resulted from differential consumption by nanoflagellates. By using relatively short incubation periods, we were able to address the question of diel changes in grazing using a dilution method and also found higher feeding rates at night than during the day (Table 1). Tsai et al. (2009) suggested that size-selective feeding was the basis of the diurnal differences in ingestion of *Synechococcus* spp. by nanoflagellates. This was based on correlation of the higher grazing rates with the shift from larger dividing *Synechococcus* cells (daytime) to smaller non-dividing cells at night (Tsai et al. 2009) and a recognition that protistan grazers can exhibit a preference for certain prey size and type (e.g. Epstein & Shiaris 1992).

We also attribute significant mortality of *Synechococcus* spp. at our study site to viruses and note a greater effect of viruses at nighttime (Table 1). The exact causes for the periodicity of viral lysis require further investigation. However, viral infection rates are density dependent (Fuhrman 1999), and the abundance of *Synechococcus* spp. was higher at nighttime than during the day (Fig. 2C). We propose that the higher *Synechococcus* spp. abundance led to increased viral infection and lysis at night. The lower *Synechococcus* spp. abundance (average of 2.2×10^4 cells ml^{-1}) during the daytime of our study raises the possibility that the population size was below a threshold host concentration necessary for viral infection, as was suggested for other populations of *Synechococcus* spp. by Suttle & Chan (1994). Threshold host abundances for successful virus infection have also been proposed for bacteria (Steward et al. 1992, Weinbauer & Peduzzi 1994) and the coccolithophore *Emiliania huxleyi* (Jacquet et al. 2002). Furthermore, a previous study showed that the length of the lytic cycle (turnover time) is usually tightly coupled with the growth rate of the bacteria (Weinbauer 2004). In the present study, viral lysis was significantly and positively correlated with *Synechococcus* spp. growth rate (data not shown), and the effects of diel variations of *Synechococcus* spp. growth rate on viral lysis cannot be excluded.

Another non-exclusive factor contributing to diel differences in *Synechococcus* spp. mortality is ultraviolet radiation (UVR). UVR could have direct effects on *Synechococcus* spp., but it also causes significant damage to viruses (e.g. Winter et al. 2004, Yuan et al. 2011) and inhibits protistan feeding (Ochs 1997). Because UVR-induced damage decreases with diminishing daylight, its inhibition of viruses and grazing are also likely to decrease towards nighttime.

The interactions between viruses and grazers and their effects on picoplankton are probably very complex (Miki & Jacquet 2008) and could include various antagonistic or synergistic effects (Sime-Ngando & Pradeep Ram 2005). For example, nanoflagellates can directly reduce viral abundance and infectivity through direct consumption of viruses or by grazing preferentially on viral-infected cells (Bettarel et al. 2005). Though our study did not directly address these complexities, it does suggest that protistan grazing and viral infections interact to modify picoplankton primary production in an oligotrophic environment.

Our data reinforce the view that the abundance of *Synechococcus* spp. exhibited a clear diel pattern during summer in the western subtropical Pacific Ocean. The results confirmed that nanoflagellate grazing was a significant cause of *Synechococcus* spp. mortality, but viral lysis was also an important source of mortality, especially at nighttime. Future research is necessary to further evaluate the ecological importance and interactions of viral lysis versus grazing as agents of diel periodicity in picophytoplankton biomass and production in the subtropical marine environment.

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