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Seasonal variations of viral- and nanoflagellate-mediated mortality of heterotrophic bacteria in the coastal ecosystem of subtropical Western Pacific

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Abstract

Since viral lysis and nanoflagellate grazing differ in their impact on the aquatic food web, it is important to assess the relative importance of both bacterial mortality factors. In this study, an adapted version of the modified dilution method was applied to simultaneously estimate the impact of both viral and nanoflagellate grazing on the mortality of heterotrophic bacteria. A series of experiments was conducted monthly from April to December 2011 and April to October 2012. The growth rates of bacteria we measured ranged from 0.078 h^{-1} (April 2011) to 0.42 h^{-1} (September 2011), indicating that temperature can be important in controlling the seasonal variations of bacterial growth. Furthermore, it appeared that seasonal changes in nanoflagellate grazing and viral lysis could account for 34 % to 68 % and 13 % to 138 % of the daily removal of bacterial production, respectively. We suggest that nanoflagellates grazing might play a key role in controlling bacterial biomass and might exceed the impact of viral lysis during the summer period (July to August), because of the higher abundance of nanoflagellates at that time. Viral lysis, on the other hand, was identified as the main cause of bacterial mortality between September and December. Based on these findings in this study, the seasonal variations in bacterial abundance we observed can be explained by a scenario in which both growth rates and loss rates (grazing + viral lysis) influence the dynamics of the bacteria community.

1 Introduction

It is well recognized that both viral lysis and protozoan grazing are important top-down controls of bacterial mortality in oceanic systems (Pace, 1988; Wilhelm et al., 2002; Taira et al., 2009). When bacterial cells are grazed, energy is made available to higher trophic levels, whereas when they are lysed, organic carbon and nutrients are dissolved and bacterial carbon is recycled as bacterial production in a closed loop (Midelboe et al., 1996; Wilhelm et al., 2002). Viruses have been shown to account for

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5 a high percentage of bacterial mortality in some marine environments (Weinbauer and Peduzzi, 1995; Wells and Deming, 2006; Taira et al., 2009), with percentages similar those due to nanoflagellates when both sources of bacterial loss have been measured simultaneously (Fuhrman and Noble, 1995; Steward et al., 1996). Therefore, it is im-
portant to estimate both viral- and grazing-induced bacterial mortality to obtain better insight into the forces underlying nutrient flow within an aquatic food web.

Most studies previously examining the impact of grazing versus viral lysis have fo-
cused on bacterial populations (Weinbauer and Peduzzi, 1995; Steward et al., 1996;
Weinbauer and Hofle, 1998; Pedros-Alio et al., 2000; Jacquet et al., 2005; Fischer
10 et al., 2006; Well and Deming, 2006). These studies have shown that both viral lysis
and grazing can cause significant mortality, but that the impact of each varies by sea-
son, host organism, or environmental condition. The importance of viral lysis has been
shown to increase in situations where protozoan grazing is reduced (Bettarel et al.,
2004). For example, Weinbauer and Höfle (1998) reported lytic mortality to be strongly
15 dominant in the anaerobic hypolimnion layer of Lake Plusssee, Germany, where protists
are scarce due to oxygen depletion. Seasonally, viral lysis is reported to be the main
cause of bacterial mortality during the winter, while grazing is reported to be the cause
of most bacterial mortality during the spring (Well and Deming, 2006; Tijdens et al.,
2008). Furthermore, nanoflagellates have been reported to be the predominant cause
20 of mortality and viruses a less important one in a summer study of a eutrophic bay (Choi
et al., 2003). To date, studies in oligotrophic waters of subtropical Western Pacific have
mainly focused on bacterial mortality due to nanoflagellate grazing (Tsai et al., 2005,
2008, 2011). The contribution of viruses to bacterial mortality in this environment of
subtropical Western Pacific is still poorly understood.

25 Experimental tests of long-term seasonal variations comparing the effect of grazing
with that of viral lysis on bacterial production are scarce in marine systems. Some pre-
vious studies have performed simultaneous monitoring and experiments to assess the
impact of viruses and nanoflagellates on heterotrophic bacterial, cyanobacteria and
phytoplankton in aquatic environments (marine or lake waters) but only for restricted

periods of the year (Evans et al., 2003; Jacquet et al., 2005; Well and Deming, 2006; Kimmance et al., 2007; Tjldens et al., 2008). Furthermore, while several long-term seasonal studies to estimate grazing and viral mortality of bacteria in freshwater environments (Personnic et al., 2009), little is known about the seasonal variations of mortality impact of viral and grazing on bacteria in marine ecosystems.

In this study, we used the modified dilution approach to estimate grazing and viral mortality of bacteria and compared their relative contributions of both on bacterial mortality for about 2 yr in the coastal ecosystem of subtropical Western Pacific. Our hypothesis was that viruses or nanoflagellates may play a significant role in controlling the seasonal variations of bacterial production, as grazing has been reported to be the cause of most bacterial mortality during the summer (Tsai et al., 2011).

2 Material and methods

2.1 Sampling

Research was conducted aboard the R/V *Ocean Research II* and Taiwan coastal water samples were collected monthly at the same time of day (1000 to 1100 h) for about 2 yr (Fig. 1). Seawater from 5 m depth was collected in a 10 l Niskin bottle and gently siphoned into clear, light-proof, 5 l polypropylene carboys. Continuous measurements of temperature and salinity were taken at the station at different levels from the surface to near bottom using a SeaBird CTD-General Oceanic Rosette. Nutrients of seawater samples were measured as previously described by Gong et al. (1995). Water samples were filtered (25 mm GF/F) for chl *a* analysis and measured after extraction with an in vitro fluorometer (Turner Design 10-AU-005) (Parsons et al., 1984). Primary productivity of surface water (5 m) was measured by ^{14}C assimilation (Parsons et al., 1984).

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2.2 Dilution experiments

We estimated viral lysis and nanoflagellate grazing rates using a modified technique involving 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” diluents, the natural sample was passed through 10 µm mesh and then filtered through a 47 mm Nuclepore filter (type PC, pore size of 0.2 µm). The filtered seawater sample (< 10 µm) was then diluted with the 0.2 µm filtered seawater in a 4-point dilution series: 25, 50, 75, and 100% seawater (< 10 µm). The mixtures were incubated for 12 h in triplicate in 50-ml polycarbonate bottles under natural light in a water bath set at the same temperature as the seawater at the time of sampling. 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 additional dilution series, which used 30 kDa filtered seawater instead of 0.2 µm filtered water, was used to modify both grazing and viral mortalities. The net growth rate of bacteria (k , h^{-1}) was calculated for each sample based on microscopic cell counts at the start and the end of the experiment (N_t and N_{t0}), assuming exponential growth (Landry and Hassett, 1982):

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

The regression coefficient of apparent growth rate versus dilution factor for the 0.2 µm dilution series, though usually interpreted as protistan grazing (mg) (Landry and Hassett, 1982), actually includes viral mortality (mv), since most viruses pass through a 0.2 µm pore (Evans et al., 2003). However, when virus-free seawater (30 kDa filters) is used as a diluent, the regression reflects release from both grazing and viral mortality (mg + mv), and a direct estimate of viral mortality for bacteria can be obtained from the difference in slopes of the regression lines between the two dilution series.

A carbon budget was determined by combining the cellular carbon content estimates and data from the modified dilution experiments. Carbon content for heterotrophic

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bacteria was based on values reported in Lee and Fuhrman (1987) (20 fgC cell^{-1}). For bacteria in this study, carbon production (BP in $\mu\text{gCl}^{-1} \text{d}^{-1}$), losses due to grazing (G , $\mu\text{gCl}^{-1} \text{d}^{-1}$), and viral lysis (V , $\mu\text{gCl}^{-1} \text{d}^{-1}$) were calculated using the following formulae: $\text{BP} = \mu \times B_0$, $G = \text{mg} \times B_0$, and $V = \text{mv} \times B_0$, where μ is the dilution-based specific growth (y-intercept of the 30 kDa regression), mg and mv are the dilution-based grazing and viral lysis rates, and B_0 is the heterotrophic bacterial biomass (μgCl^{-1}) at the sampling time.

2.3 Viral, bacterial, *Synechococcus* spp. and nanoflagellate abundance counts

Viruses, bacteria, *Synechococcus* spp. and nanoflagellates were counted using an epifluorescence microscope (Nikon Optiphot-2) (1000 \times). Viruses were processed using a slight modification of a protocol described by Nobel and Fuhrman (1998). Briefly, samples from 0.5 to 1 ml were filtered on Anodisc filter (0.02 μm pore size, Whatman) backed by 0.45 μm pore size Millipore filter. 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 placed on glass slides and added with 25 μl of 50 % glycerol/ 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 1–2 ml or 20 ml were filtered onto 0.2 μm or 0.8 μm black Nuclepore filters for picoplankton (bacteria and *Synechococcus* spp.) and nanoflagellates, respectively. Samples were stained with DAPI at a final concentration of 1 $\mu\text{g ml}^{-1}$ (Porter and Feig, 1980) to count bacteria and heterotrophic nanoflagellates (HNF). Pigmented nanoflagellates (PNF) and HNF were counted based on the absence or presence of chlorophyll autofluorescence using a separate filter set optimized for chlorophyll or DAPI under a 1000 \times epifluorescence microscope (Nikon-Optiphot-2). Bacteria and HNF were identified by their blue fluorescence under UV illumination. PNF and *Synechococcus* spp. were identified by their red and orange autofluorescence under blue excitation light. To obtain reliable estimates of abundance we counted 30, 30,

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30 and 50 fields of view for viruses, bacteria, *Synechococcus* spp. and nanoflagellates, respectively.

2.4 Statistical analysis

Least-square regression analysis was performed to analyze the relationship between bacterial growth rate and fraction of 30 kDa or 0.2 μm dilution series. Significance of the regression lines was tested using an analysis of variance (ANOVA). Moreover, the significance between the slopes of the 30 kDa and 0.2 μm dilution series was determined using an *F*-test. If the regression slopes of 30 kDa and 0.2 μm dilution series were significantly different, we calculated the magnitude of viral mortality. STATISTICA 7.0 software was used for all statistical operations. A probability value of < 0.05 was considered significant.

3 Results

3.1 Physical, chemical and biological characterization of the study area

Surface water temperatures during the study showed strong seasonality with maximum values recorded during the summer period (32°C in June 2012) and minimum values during winter (18.4°C in December 2011) (Table 1). Chlorophyll *a* concentrations and primary productivity also showed distinct seasonal patterns with highest values recorded in summer (2.41 mgm^{-3} and $320.72\text{ mgCm}^{-3}\text{d}^{-1}$ in July 2011) and lowest in cold season ($< 20^\circ\text{C}$, 0.31 mgm^{-3} in April and $6.29\text{ mgCm}^{-3}\text{d}^{-1}$ in December 2011) (Table 1).

The results of the seasonal variations in bacterial, *Synechococcus* spp. and viral abundance are shown in Fig. 2a. Abundance of bacteria and *Synechococcus* spp. during the study period ranged from 4.1×10^5 to $8.6 \times 10^5\text{ cellsml}^{-1}$ and 0.6×10^4 to $5.1 \times 10^4\text{ cellsml}^{-1}$, respectively (Fig. 2a). Furthermore, viral abundance was found to range from 2.1 to $9.2 \times 10^6\text{ viruses ml}^{-1}$ and was found to demonstrate a clear pattern in

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seasonal variability at this study site (Fig. 2a). Seasonal variations of PNF abundance were similar to that of HNF throughout the study period, though HNF abundance was lower than PNF in the summer (Fig. 2b).

According to our data, viral abundance increased with increasing bacterial numbers and Chl *a* concentrations, exhibiting a significant relationship among of them ($r = 0.75$, $p < 0.05$; $r = 0.58$, $p < 0.05$) (Fig. 3a, b). Furthermore, the relationship between *Synechococcus* spp. and viral abundance was also significant in our direct counts ($r = 0.45$, $p < 0.05$) (Fig. 3c).

3.2 Dilution experiments and bacterial growth and mortality

We used a modified dilution method to estimate rates of bacterial growth (μ), grazing (mg) and viral lysis (mv) from April to December 2011 and April to October 2012 (Table 1). Regression analysis was applied to the dilution experiments, which allowed us to determine both the growth and mortality coefficient in each of parallel dilution series (Table 1). For 26 of the 28 experiments, there was a significant relationship ($p < 0.05$) between bacterial growth rate and the level of dilution in both the 0.2 μm and 30 kDa series (Table 1). However, there was no significant difference (F -test, $p > 0.05$) between the regression slopes of growth rate and level of dilution of the 0.2 μm and 30 kDa series in June, July and August 2012 experiment (Table 1). For this situation, an estimate of viral mortality of bacteria at a level of significance of $p < 0.05$ could not be determined for this experiment (Table 2).

The y-intercepts of these regression lines for 30 kDa series represent the growth rates of bacteria in the absence of lytic and grazing pressure. They ranged from 0.078 h^{-1} (April 2011) to 0.42 h^{-1} (September 2011) (Table 2). Moreover, our study shows that temperature can play an important role in controlling bacterial growth, as we found there to be positive relationships between the growth rates and temperature (growth rate (h^{-1}) = $0.017 \text{ temp} - 0.29$, $r^2 = 0.54$, $p < 0.05$) (data not shown). Furthermore, the regression coefficients (slopes) for 0.2 μm fractionated series, which represent bacterial grazing mortality (mg), ranged from 0.033 to 0.18 h^{-1} (Table 2). We found

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seasonal variation in viral lysis rates, with slope differences between 0.2 μm and 30 kDa series ranging from 0.038 h^{-1} to 0.300 h^{-1} during the study period (Table 2).

Our results also showed that the carbon losses due to viral lysis and nanoflagellate grazing were 8.55–64.80 $\mu\text{gCl}^{-1}\text{d}^{-1}$ and 9.79–118.08 $\mu\text{gCl}^{-1}\text{d}^{-1}$, respectively (Table 2). It appeared that nanoflagellate grazing and viral lysis could account for 33.3 % to 66.7 % and 13.1 % to 137.8 % of the daily removal of bacterial production, respectively (Fig. 4). The impact of viruses on bacterial mortality had not been measured between June and August 2012 (Fig. 4). We found a negative linear relationship between the bacterial abundance and the total carbon losses generated by nanoflagellate grazing and viral lysis ($r^2 = 0.50$, $p < 0.05$) (Fig. 4).

4 Discussions

The present study explored seasonal variations in bacterial mortality caused by grazing and viruses in the coastal ecosystem of subtropical Western Pacific. The ratio of total mortality generated by nanoflagellate grazing were found to be generally higher in summer periods (July 2011), especially between June and August 2012, when no significant viral lysis rates were recorded. However, viral lysis was found to be the main cause of bacterial mortality between September and December, when it removed between 53 % and 137 % of the potential bacterial production (Fig. 4).

Previous work in the same region, which used size-fractionation approach with 2 μm filters, reported the average bacterial growth to be 0.046 h^{-1} in the summer (Tsai et al., 2008). In the current study, which followed standard protocol with 0.2 μm diluents, the growth rates of bacteria ranged from 0.084 to 0.094 h^{-1} in the summer periods (June–October) (Table 1), about 2-fold greater than previous reported by Tsai et al. (2008). Both of these methods have a degree of error associated with them, because not all nanoflagellates may be eliminated using 2 μm filters. About 12 % of nanoflagellates pass through a 2 μm filters in the size-fractionation approach (Tsai et al., 2008). This situation could have caused an underestimation of bacterial growth rates. Moreover,

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the current study observed higher bacterial growth rates in the 30 kDa (grazer and virus-free water) than in the 0.2 μm diluents (Table 1), suggesting that under conditions in which lytic pressure was relatively high, the growth rate obtained from the standard protocol (0.2 μm series) might have been underestimated because almost all viruses could remain in the 0.2 μm diluents water samples. Similar findings have been recorded (Well and Deming, 2006; Tijden et al., 2008; Taira et al., 2009).

To understand the role of viruses in the marine environment, it is important to know the seasonal changes of viral abundance in relation to the variations of bacteria, phytoplankton and other biological parameters. We observed positive correlations were observed between viruses and bacteria. Similar relations between viruses and bacteria have also been reported in most aquatic environments (Alonso et al., 2001; Hewson et al., 2001; Bettarel et al., 2003), where almost all of the viruses seem in fact to be bacteriophages (Wommack and Colwell, 2000). Based on a linear regression analysis in this study, variation in bacterial abundance was found to explain 56 % of the variability in viral abundance and could be used as the sole predictor to explain seasonal change in virus abundance and be a better degree of explanation than Chl *a* ($r^2 = 0.34$). These results are similar to those reported by Cochlan et al. (1993), who demonstrated that bacterial abundance could be superior predictor variable to chlorophyll *a*. In their models, bacterial abundance explained 69 % of the variation in viral abundance as opposed to chlorophyll, which explained only 45 %. The generally poorer correlations between virus abundance and chl *a* concentration does not discount the possibility that phytoplankton are significant host of the viruses at our study site. Since chl *a* is only a proxy for phytoplankton biomass, it may not accurately reflect phytoplankton abundance. Furthermore, previous studies on viral lysis in oligotrophic environments have focused on *Synechococcus* spp. (Garza and Suttle, 1998; Baudoux et al., 2007, 2008), though the present study found a poorer correlation between viruses and *Synechococcus* spp. abundance (Fig. 3c). The poorer correlation between viruses and *Synechococcus* spp. abundance might suggest that *Synechococcus* spp. has distinct diel changes in abundance during summer, with higher abundance at nighttime. Furthermore, viral lysis is

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reported to be an important cause of *Synechococcus* spp. mortality, especially at night-time (Tsai et al., 2012). Viral dynamics might be controlled by external environmental factors, such as temperature and light. The fact that no diel rhythm in viral abundance was observed in the present study, however, viral infectivity was sensitive to solar UV radiation (Suttle and Chen, 1992; Wilhelm et al., 1998; Hofer and Sommaruga, 2001). Suttle and Chen (1992) suggest that there should be a strong diel signal in the abundance of infective viruses. Temperature can also be involved in controlling the abundance of viruses as it temperature controls bacterial growth rates and has a significant positive effect on bacterial production (Shiah and Ducklow, 1994; Tsai et al., 2008). Jiang and Paul (1994) found a positive correlation between viral abundance and temperature, as we did in the current study. ($r = 0.4$, $p < 0.05$.)

The seasonal abundances of viruses and bacteria in this study were rather homeostatic as they did not vary by more than 4-fold during the study periods. This contrasts with the seasonal variation we found in the abundances of nanoflagellates, which varied by up to 6-fold for HNF, and 10-fold for PNF. In marine environments, it is common to have variations in the abundance of viruses between winter and summer of greater than one order of magnitude (Jiang and Paul, 1994; Weinbauer et al., 1995). One previous study showed that an increase in viral abundance is generally observed with increasing trophic status (Wommack and Colwell, 2000). In Jiang and Paul (1994), the highest value of chlorophyll *a* was 12.8 mg m^{-3} , which was higher than the summer values ($0.8\text{--}2.4 \text{ mg m}^{-3}$) at our study site. Bettarel et al. (2003) also reported there to be an increasing relative abundance of such non-bacteriophage viruses as cyanophages in productive environments. This finding may explain why the seasonal abundance of viruses was significantly less varied in this study than the abundances reported in other studies. Furthermore, a particularly interesting finding in this study was that there was a more pronounced increase in abundance of PNF than in HNF during the summer period (Fig. 2b), which is similar to a study by Tsai et al. (2010), who reported that the increase in PNF caused the variation of nanoflagellate community of the East China Sea in the summer. Similar observations have been reported in other studies

(Sanders et al., 1992; Tamigneaux et al., 1995), suggesting that PNF often shows a more pronounced seasonal pattern than HNF in response to change in light and temperature. Furthermore, the interactions between viruses and grazers and their effects on picoplankton are probably very complex (Miki and Jacquet, 2008) and could include various antagonistic or synergistic effects (Sime-Ngando and 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 bacterial production.

To the best of our knowledge, the results of this study provide a first estimation of the potential effects of viral lysis and protozoan grazing on bacteria in this area. Several methods have been used to estimate viral mortality on components of the microbial food web. These include tracking changes in viral abundance, production or decay rate (Heldal and Bratbak, 1991; Wilhelm et al., 2002) and measuring the frequency of virus-infected bacterial cells (Proctor et al., 1993; Binder, 1999). These methods are based on several assumptions about latent periods and/or burst sizes for viruses. One advantage of the modified dilution method used in this study is that it provides insight into the quantitative significance of both viral lysis and grazing without the need of conversion factors (Jacquet et al., 2005). This dilution technique is now commonly applied to a wide variety of prey and environments (Weisse and Scheffel-Moeser, 1990; Worden and Binder, 2003). We also wanted to estimate mortality due viral lysis. To do this, we used the additional dilution series method (Evans et al., 2003), which has been successfully used to estimate the impact of viral lysis on the haptophyte *Phaeocystis globosa* (Scherffel) in temperate coastal waters (Baudoux et al., 2006) and the impact of viral lysis on bacterioplankton in freshwater and oceanic waters (Jacquet et al., 2005; Well and Deming, 2006; Tjeldens et al., 2008; Taira et al., 2009).

Generally, heterotrophic bacterial abundance and productivity in aquatic environments may be linearly dependent on phytoplankton biomass and productivity (Gasol

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et al., 1998). In many oligotrophic waters, there is a close association between heterotrophic bacterial and phytoplankton production (Gasol and Duarte, 2000; Duarte and Agustí, 2005), indicating that primary production ultimately provides most of the carbon needed for heterotrophic bacterial production in these settings. However, as shown in Fig. 5, this study found a significant relationship between heterotrophic bacterial growth rates and released dissolved organic carbon by lysis (V), but no significant relationship between heterotrophic bacterial growth rates and primary production ($\rho = 0.1$). This observation suggests that viral infection is an important mechanism in C recycling in the sea. Similarly, culture studies by Middleboe et al. (1996) demonstrate a tight coupling between phosphorus released by viral lysis of bacteria and bacterial growth. In fact, viruses cause the death of their host cells (Fuhrman and Noble, 1995; Steward et al., 1996), and this viral activity produces dissolved organic carbon from lysis of particles and thereby promotes the recycling of carbon by bacteria in the water column (Nobel et al., 1999; Middelboe and Lyck, 2002). These results suggested that viral lysis of bacteria can result in both a sudden and large release of dissolved organic carbon and a rapid increase in bacterial growth rates.

Large seasonal variations in microbial mortality have been recorded in other environments (Domaizon et al., 2003; Ortmann et al., 2011). Based on the dilution experiments in the current study, the ratio of seasonal variations of grazing effect (mg) to total mortality (mg + mv) changed from 21 to 76 % (Table 2). The ratios of total mortality generated by nanoflagellate grazing were generally higher in summer periods (July 2011), especially between June and August 2012, with no significant viral lysis rates recorded during these periods (Table 2). We suggest that nanoflagellate grazing could play a key role in controlling bacterial biomass and might exceed the impact of viral lysis during summer period. Personnic et al. (2009) determined that nanoflagellate grazing was a significant cause of mortality in heterotrophic bacteria in the autumn (up to 42 %) and summer (up to 76 %), but in the winter and early spring, which are periods of relatively low productivity, neither the impact of the viruses nor that of the nanoflagellates could be clearly detected. The distinction and quantification of bacterial

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losses due to lysis and nanoflagellate grazing is essential for an optimal understanding of the carbon pathway in marine environments. Nanoflagellate grazing will mostly channel bacterial biomass to higher trophic levels in the microbial food web. Through cell lysis, bacterial biomass is converted to dissolved organic carbon, hence forcing the food web towards a more regenerative pathway (Wilhelm and Suttle, 1999). Our results substantiate earlier studies suggesting that nanoflagellate plays an important role in bacterial carbon transfer to higher levels of the food web at this study site during summer (Tsai et al., 2011). However, bacterial production was not balanced by nanoflagellate grazing and viral lysis between June and August (33–68 % of bacterial production) (Fig. 4); therefore, other sources of bacterial losses, including cell death or sedimentation (Pace, 1988), may account for the imbalance. Simek et al. (1990) observed that ciliates contributed an average of 71 % to the protozoan bacterivory and balanced bacterial production in the summer period. The importance of ciliates in our study could not be determined because their numbers are so low (Chen, 2003). Chen (2003) also reported that ciliates account for the removal of only about 3 % of *Synechococcus* spp. production. Thus, the potential effect of nanoflagellate predation on the removal of picoplankton in our study appears to be substantial.

In fact, seasonal cycles of bacterial abundances are a reflection of the changing net growth rates (growth rate-loss rates). As shown in Fig. 4, total loss rates accounted for 33–68 % of bacterial production (positive net growth rates) occurring from June to August. During this period, the abundance of bacteria sharply increased. The total loss rate (grazing + viral lysis) gradually increased, accounting for 105 %–174 % of bacterial production between September and December. During this period, temperature dropped after September, the net growth rates were negative, and bacterial abundance declined (Fig. 2a). Based on these findings, the observed seasonal variations in bacterial abundance can be explained by a scenario in which both growth rates and loss rates (grazing + viral lysis) influence the dynamics of the bacteria community. With regard to seasonal changes of viral lysis on bacterial mortality, viral lysis was identified as the main cause of bacterial mortality September and December, when it clearly

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removed between 53% and 137% of the potential bacterial production (Fig. 4). This result similar to other studies (Jacquet et al., 2005; Tijdens et al., 2008), which showed viral lysis to be the main cause of bacterial mortality during cold season experiments. Jacquet al. (2005) also observed that during the January experiment viral lysis removed up to 100% of the potential bacterial production.

5 Conclusions

Results from the present study reveal that a modified dilution approach can be adapted to directly determine virus-induced mortality rates of bacteria in the coastal ecosystem of subtropical Western Pacific. In this study, the ratio of total mortality generated by nanoflagellate grazing were generally higher in summer periods. We conclude that grazing transfers production from picoplankton to higher trophic levels, rather than shunting it into the dissolved pool through viral lysis and that viral lysis did the main cause of bacterial mortality between September and December, when it removes between 53% and 137% of the potential bacterial production. Further studies are recommended to assess the wider applications of the dilution protocol developed in this study for diel variations.

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Table 1. Environmental factors (temperature, chlorophyll *a* and primary production) and results from the parallel dilution experiments. The significance (*p*) of the regression analyses (ANOVA) and the significance between the slopes of the regression of 0.2 μm and 30 kDa dilution series determined using an *F*-test are shown. ND: no data.

2011	<i>T</i> (°C)	chl <i>a</i> (mgm^{-3})	PP ($\text{mgCm}^{-3}\text{d}^{-1}$)	Diluent	Regression equation	Regression (<i>p</i>)	Difference of regression slopes (<i>p</i>)
Apr	19.7	0.31	50.28	0.2 μm	$y = -0.037x + 0.045$	< 0.05	< 0.01
				30 kDa	$y = -0.075x + 0.078$	< 0.01	
May	21.1	0.43	14.84	0.2 μm	$y = -0.039x + 0.052$	< 0.01	< 0.05
				30 kDa	$y = -0.079x + 0.083$	< 0.01	
Jun	ND	ND	ND	0.2 μm			
				30 kDa			
Jul	27.5	2.41	320.72	0.2 μm	$y = -0.148x + 0.094$	< 0.01	< 0.05
				30 kDa	$y = -0.195x + 0.36$	< 0.01	
Aug	ND	ND	ND	0.2 μm			
				30 kDa			
Sep	28.1	0.44	53.20	0.2 μm	$y = -0.142x + 0.084$	< 0.01	< 0.01
				30 kDa	$y = -0.445x + 0.42$	< 0.01	
Oct	25.3	0.8	12.26	0.2 μm	$y = -0.18x + 0.093$	< 0.01	< 0.05
				30 kDa	$y = -0.325x + 0.27$	< 0.01	
Nov	21	0.55	18.42	0.2 μm	$y = -0.099x + 0.046$	< 0.01	< 0.01
				30 kDa	$y = -0.2x + 0.17$	< 0.01	
Dec	18.4	0.32	6.29	0.2 μm	$y = -0.033x + 0.038$	< 0.05	< 0.01
				30 kDa	$y = -0.157x + 0.146$	< 0.05	
2012							
Apr	24.5	ND	ND	0.2 μm	$y = -0.045x + 0.052$	< 0.01	< 0.01
				30 kDa	$y = -0.096x + 0.095$	< 0.05	
May	25.4	ND	ND	0.2 μm	$y = -0.043x + 0.055$	0.064	< 0.05
				30 kDa	$y = -0.131x + 0.158$	< 0.01	
Jun	32	ND	ND	0.2 μm	$y = -0.12x + 0.23$	< 0.05	0.326
				30 kDa	$y = -0.16x + 0.28$	< 0.05	
Jul	28.5	ND	ND	0.2 μm	$y = -0.082x + 0.048$	< 0.01	0.298
				30 kDa	$y = -0.106x + 0.121$	< 0.01	
Aug	26.6	ND	ND	0.2 μm	$y = -0.05x + 0.038$	< 0.01	0.18
				30 kDa	$y = -0.092x + 0.15$	< 0.01	
Sep	27.9	ND	ND	0.2 μm	$y = -0.08x + 0.24$	0.063	< 0.05
				30 kDa	$y = -0.18 + 0.31$	< 0.05	
Oct	23.5	ND	ND	0.2 μm	$y = -0.05x - 0.071$	< 0.05	< 0.05
				30 kDa	$y = -0.142 + 0.127$	< 0.05	

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Table 2. Dilution-based specific growth rates in 30 kDa experiments (μ), nanoflagellate grazing (mg) and viral lysis rates (mv) for the heterotrophic bacteria. Daily bacterial carbon production (BP) and the fraction of carbon loss by nanoflagellate grazing (G), viruses (V) and the ratios of nanoflagellate grazing (mg) to total mortality of bacteria (mg + mv) was calculated for each experiment. ND: no data, – : grazing or viral lysis estimates were not statistically significant ($p > 0.05$).

	μ (30 kDa) (h ⁻¹)	mg (h ⁻¹)	mv (h ⁻¹)	BP ($\mu\text{gCl}^{-1}\text{d}^{-1}$)	G ($\mu\text{gCl}^{-1}\text{d}^{-1}$)	V ($\mu\text{gCl}^{-1}\text{d}^{-1}$)	mg (%) (mg mg ⁻¹ + mv) × 100 %
2011							
Apr	0.078	0.037	0.038	22.46	10.66	10.94	49 %
May	0.083	0.039	0.040	25.10	11.79	12.10	49 %
Jun	ND	ND	ND	ND	ND	ND	ND
Jul	0.360	0.148	0.047	136.51	56.12	17.82	76 %
Aug	ND	ND	ND	ND	ND	ND	ND
Sep	0.420	0.142	0.300	165.31	55.89	118.08	32 %
Oct	0.270	0.180	0.145	97.20	64.80	52.20	55 %
Nov	0.170	0.099	0.101	62.83	36.59	37.33	50 %
Dec	0.090	0.033	0.124	23.33	8.55	32.14	21 %
2012							
Apr	0.095	0.045	0.051	19.61	9.29	10.53	47 %
May	0.158	–	0.131	38.68	–	9.79	–
Jun	0.280	0.120	–	102.14	43.78	–	–
Jul	0.121	0.082	–	37.17	25.19	–	–
Aug	0.150	0.050	–	72	24	–	–
Sep	0.310	–	0.180	117.55	–	68.26	–
Oct	0.127	0.050	0.092	37.80	14.88	27.38	35 %

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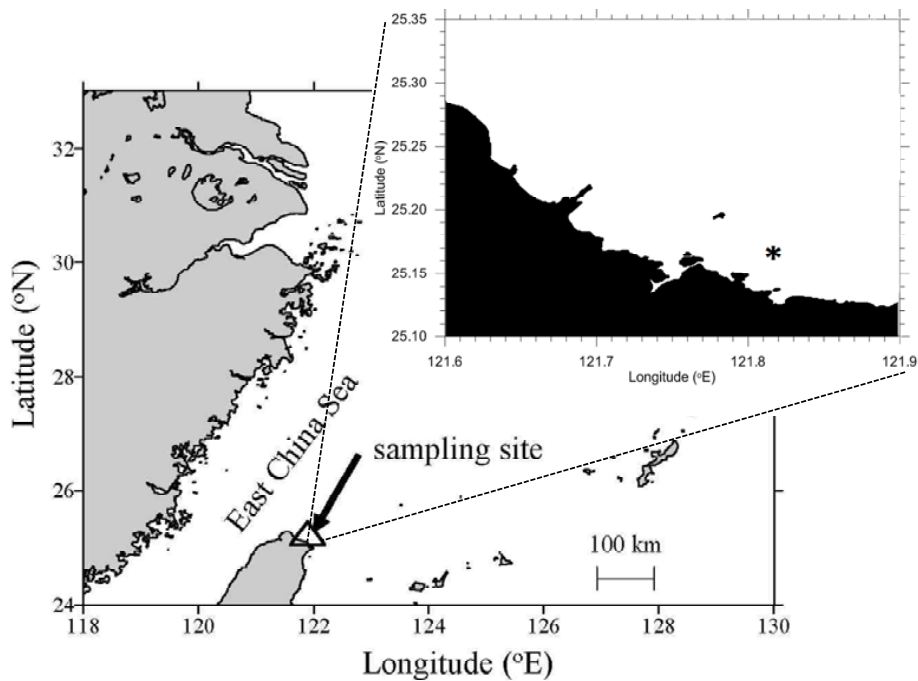
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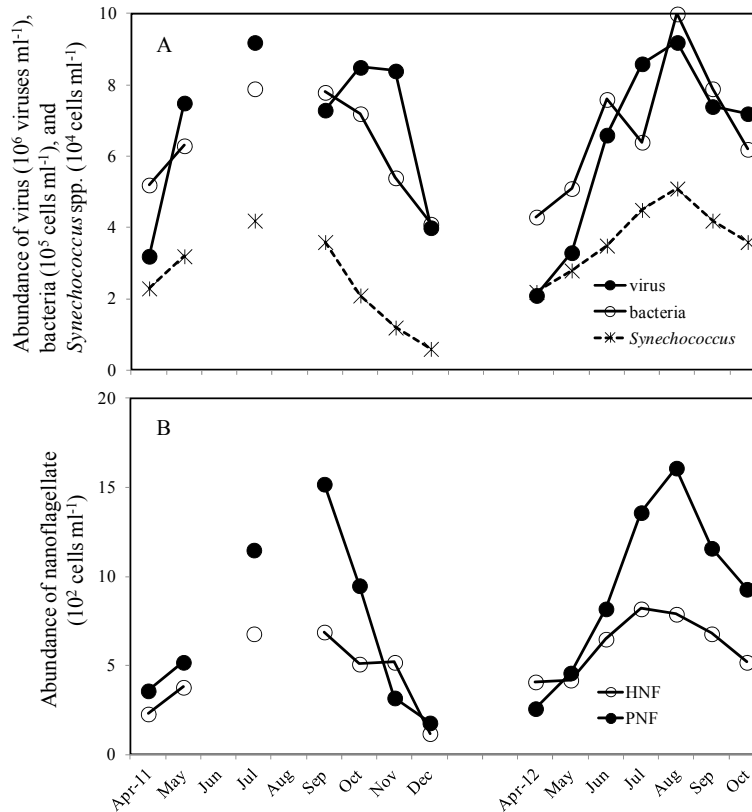


Fig. 2. Seasonal variations of bacterial (10^5 cells ml^{-1}), viral (10^6 viruses ml^{-1}) and *Synechococcus* spp. abundance (10^4 cells ml^{-1}) (A) and heterotrophic nanoflagellate (HNF) (10^2 cells ml^{-1}) and pigmented nanoflagellate (PNF) (10^2 cells ml^{-1}) (B) of surface water (5 m) during the study period.

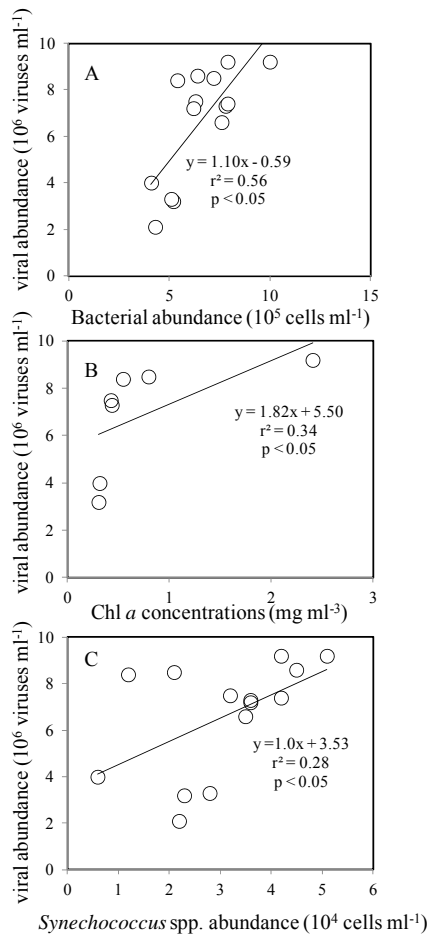


Fig. 3. Relationships between viral and bacterial **(A)**, chl *a* concentrations **(B)** and *Synechococcus* spp. abundance **(C)**.

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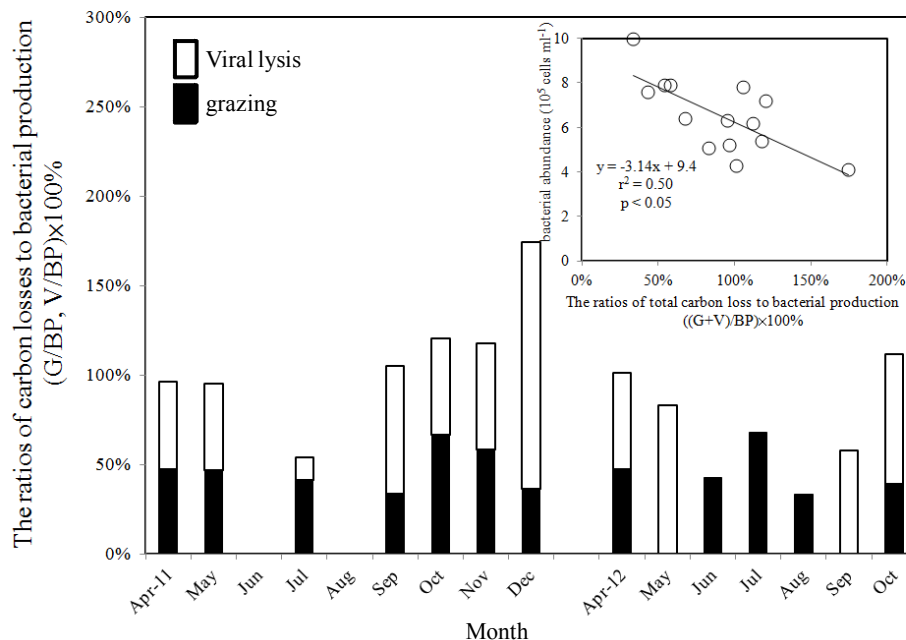


Fig. 4. Seasonal variations in the ratios of carbon losses by nanoflagellate grazing (■) and viral lysis (□) to bacterial production (BP) during the study period. The smaller figure in Fig. 4 shows the relationship between the ratios of total carbon losses to bacterial production (BP) and bacterial abundance.

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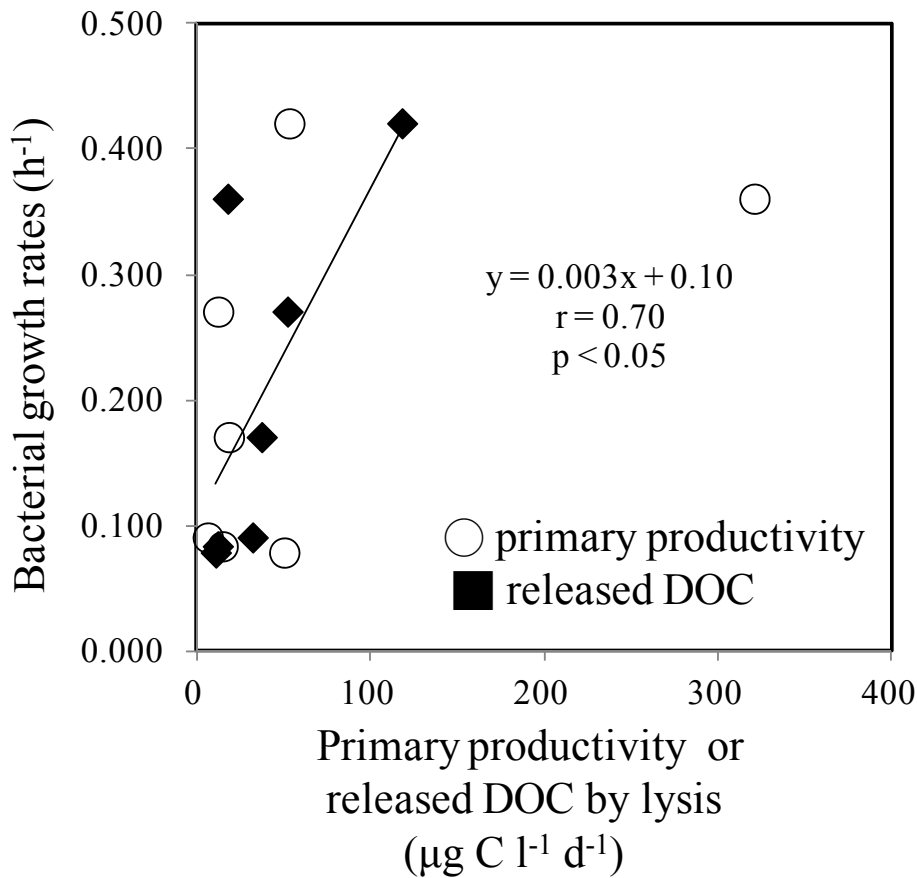


Fig. 5. Relationship between bacterial growth rates and primary production and released organic carbon (DOC) by viral lysis on bacteria (V).