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Diel Variation of Viral Production in a Coastal Subtropical Marine System

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Abstract: Viral production (VP) and bacterial mortality by viral lysis critically influence the production and mortality of aquatic bacteria. Although bacterial production, mortality by viral lysis, and viral density have been found to exhibit diel variations, the diel change in viral production has rarely been investigated. In this study, we conducted two diel dilution incubation experiments in a semi-enclosed, nutrient-rich coastal region in northeastern Taiwan to estimate the diel viral production and the mortality by viral lysis. We also compared two methods (linear regression between viral density and time versus arithmetic mean of VP during incubation) of estimating viral production. We found that viral production estimated by linear regression and bacterial mortality by viral lysis were higher during the daytime than during the nighttime. A possible explanation for the high viral production at daytime is that the bacterial community was composed of cell types with higher burst sizes at daytime. We further argued that the classical linear regression method can be used only when viral density significantly linearly increases with time, which does not always occur in dilution incubations. This study offered observations of diel variation in viral dynamics and discussed the methods estimating viral production in a marine environment.

Keywords: viral production; viral lysis; dilution incubation; diel cycle; flow cytometry

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1. Introduction

Viral lysis is an important process that regulates carbon and nutrient biogeochemical cycles and energy flows in marine plankton food webs [1]. In marine ecosystems, viral density is a sublinear power-law function of bacteria density [2], and viral lysis is one main cause of mortality of bacteria and picophytoplankton. Viral lysis can trigger substantial removal of planktonic bacteria and decrease picophytoplankton production [3–5], competing with the grazing mortality by nanoflagellates. Instead of transferring energy to higher trophic levels, viral lysis recycles nutrients and organic carbon back to the microbial community, thus, limiting the energy flux and zooplankton secondary production in marine food webs [6]. Estimating lytic mortality in bacteria is thus crucial to assessing energy transfer, biogeochemical cycling, and ecosystem functioning in aquatic systems.

Microbial mortality by viral lysis and microbial production often displays a diel cycle, but how viral production varies diurnally with the dynamics of their host in nature is elusive. In subtropical seas, diurnal bacterial production increases from late afternoon, peaking at midnight and decreasing until noon [7,8]; in the Mediterranean Sea, the heterotrophic bacterial production increases in the morning, summitting before noon or in the

afternoon and lowering through the evening and night [9]. The adsorption and transcription of cyanophages also exhibit a dark–light diel rhythm [10]. Influenced by the activity of hosts, viral infection exhibits diel variation. Previous studies have reported that the abundance of virus-infected bacterial cells would increase after bacterial activity reached a peak at noon, and the release of viruses would subsequently increase in the late afternoon in the North Sea [11]. Viral production (VP, the number of viruses released from hosts in an hour), which may be influenced by the activity of microbial hosts, can exhibit a diel cycle as their host. Nevertheless, exactly how viral production varies diurnally has rarely been investigated [11,12].

In this study, we conducted dilution incubations to estimate the diurnal variation of viral production. Furthermore, we calculated the mortality of bacteria by viral lysis from viral production. We hypothesized that viral production and bacterial mortality by viral lysis exhibit diurnal change, which are connected with the diel variation in bacterial growth and activity. Our study aimed to understand the diel variation of viral production and its potential effects on microbial food chains and nutrient cycling.

2. Materials and Methods

2.1. Incubation Procedure

We estimated VP by dilution incubation [13]. Daytime incubations were conducted from 8:00 to 20:00 (seawater was collected at 6:30; sunset at 18:30), and nighttime incubations were conducted overnight from 19:00 to 7:00 (seawater was collected at 17:30; dawn at 5:40) local time on 2 and 8 September 2020. We collected surface water for incubation at a semi-enclosed coastal station in northeastern Taiwan (25°09′06.8″ N, 121°46′31.1″ E). The nitrate concentrations in this eutrophic area ranged from 5.2–143 μmol L^{-1} (average 28.9 μmol L^{-1}) [14]. The depth of the station was approximately 3 m and the influence from waves was insignificant. The main disturbance to the water column at this station were precipitation and tides. Thus, we conducted our experiments only on days without rain within one week. To eliminate the mortality by nanoflagellate grazing, the grazer-free (<2 μm) seawater was prepared by gently vacuum filtering 2 L of surface seawater through a 47 mm diameter, 2 μm pore-size polycarbonate track-etched filter membrane (Whatman). To prevent the new infection of bacterial hosts by viruses, we reduced the viral and bacterial density in incubation water (a simplified method based on Winget et al. [15]). For the dilution of viral and bacterial density, 200 mL of virus-free water was produced by filtering grazer-free seawater through a Minimate tangential flow filtration (TFF) Capsule (Pall), with a molecular weight cut-off of 30 kDa. TFF reduces the filter clogging and does not fundamentally change the dissolved carbon and nutrient composition in water, and thus this method, with small manipulation effects, should be suitable for filtering viral particles [15–17].

Dilution was performed by adding 200 mL of the virus-free water to 50 mL of grazer-free water. This simultaneously decreased the host (mainly heterotrophic bacteria) and viral density to approximately 20% to that of the original seawater, which lowered the contact possibility of viruses and host and thus hindered new infections. The diluted incubation water was thoroughly mixed and filled in three 50-mL plastic incubation tubes. Diluted seawater was incubated for 12 h in a water tank of in situ air temperature with a natural light cycle. Viral subsamples of 0.5 mL were collected every hour from each diluted incubation tube and fixed in glutaraldehyde (0.5% final concentration) at 4 °C for at least 15 min, and subsequently deep frozen in liquid nitrogen. Three subsamples (1 mL) of bacterial community were collected at the beginning of incubation from diluted and non-diluted seawater and fixed in paraformaldehyde (1% final concentration). Virus and bacterial samples were preserved at −80 °C until flow cytometry analysis.

2.2. Enumeration of Virus and Host Density by Flow Cytometry (FCM)

Viral and bacterial samples were analyzed using a CytoFLEX S flow cytometer (Beckman Coulter, Indianapolis, IN, USA) equipped with a 488 nm air-cooled argon-ion laser, a standard 525 nm filter, and an SYBR signal trigger. Viral samples were diluted at a 1:10

concentration in TE buffer (pH 8.0, EM grade) prior to staining, to minimize the interference from high particle density. The diluted samples were stained with SYBR Green I (final concentration 1:50,000 of commercial stock) and incubated in the dark at 80 °C for 10 min. After staining, samples were cooled in an ice bath to 25 °C and processed through FCM according to the method of Brussaard [18]. Blank controls of TE buffer stained with the same concentration of SYBR Green I were run for detecting and eliminating any noise from the buffer. The quantification of viruses by FCM is comparable to epifluorescence microscopy and is suitable for the fast quantification of viruses [19]. Bacterial samples were stained with SYBR Green I (final concentration 1:10,000) for 15 min in the dark and processed through FCM according to the protocol of Hammes and Egli [20]. The densities of viruses and bacteria in the incubations are presented in Table S1 (2 September 2020) and Table S2 (8 September 2020).

2.3. Calculation of Viral Production

VP (viruses mL $^{-1}$ h $^{-1}$) was calculated using two methods: (1) linear regression between virus-like particles (VLP) density and incubation time (VP-L) [21], and (2) arithmetic mean of VP during incubation (VP-M). We calculated the VP-L from the slope of the linear regression between mean VLP density and VLP density from three replicates versus incubation time by lm function in R (version 4.0.2). We calculated the VP-M from the arithmetic mean of VP during incubation following Leuf et al. [22]. First, the local minima and maxima of viral density were identified by the R function local.min.max (R package spatialEco, version 1.3–2). Within the period between each pair of local minimum and maximum when VLP monotonically increased, the local increase rate of viral density (local VP) was calculated. These local VPs were averaged to obtained VP-M. To retrieve the non-diluted viral production, we divided VP-L and VP-M calculated from dilution incubation with the dilution factor estimated from the ratio of bacterial density in diluted and non-diluted seawater. The time series of VLP density in three replicates of incubation is shown in Figure A1. Bacterial mortality by viral lysis (B_{lysis}-L and B_{lysis}-M) was calculated by dividing viral production with mean burst size in marine systems (24 viruses per cell; Parada et al. [23]). The percentage of bacteria lysed by viruses per hour (R_{lysis}-L and R_{lysis}-M) was calculated as bacterial mortality by viral lysis divided by non-diluted bacterial density in the beginning of the incubation [13]. The code for analyzing viral production and bacterial mortality is in the supplementary material EstVP_Pub.r.

3. Results and Discussion

Our study identified different patterns of viral density change with respect to time for the incubations of 2 and 8 September 2020. First, we found that the total density of heterotrophic bacteria was higher on 2 September than on 8 September, and bacterial density did not differ a lot between day and nighttime (Table 1). The actual dilution factors were approximately 22% to 33% (Table 1). On 2 September, the mean viral density significantly and linearly increased with time in both daytime and nighttime incubations, but the oscillation of viral density was slightly larger at night (linear regression $R^2 = 0.80$, $p < 0.001$ for day and $R^2 = 0.75$, $p < 0.001$ for night incubations; Figure 1A). In contrast, on 8 September, the viral density remained stable for 10 h and increased by 10-fold (from 10^6 to 10^7 mL^{-1}) at 19:00–20:00 in the end of daytime incubation (linear regression $R^2 = 0.29$, $p = 0.056$; Figure 1B), while it remained oscillating throughout the nighttime incubation (linear regression $R^2 = 0.02$, $p = 0.637$; Figure 1B). On 2 September, viral production calculated by linear regression (VP-L) was similar to the arithmetic mean of VP in net increase periods (VP-M) during daytime incubation, while VP-L was half the value of VP-M at nighttime incubation (Table 2). Daytime VP-L were significantly higher than at nighttime ($t = 6.879$, $p = 0.0139$); daytime VP-M was higher than at nighttime, but the difference was not significant ($t = -0.340$, $p = 0.753$) on 2 September. On 8 September, VP-L was much lower than VP-M in both the daytime and nighttime incubations (Table 2). The large variation of viral density in the last two hours of daytime incubation on 8 September was due to the one-hour

difference of abrupt increase in the replicates of incubation (VLP increased at 20:00 in two replicates, but at 19:00 in the other replicate; Figure A1B). Daytime VP-L and VP-M were both significantly higher than at nighttime (t = 13.553, p = 0.0053 for VP-L and t = 4.440, p = 0.0373 for VP-M) on 8 September. On 2 September, bacterial mortality by viral lysis based on VP-L and VP-M (B_{lysis}-L and B_{lysis}-M) were higher during the daytime incubation (Table 2). The percentage of bacteria lysed calculated from B_{lysis}-L (R_{lysis}-L) was higher at day, but the percentage calculated from B_{lysis}-M (R_{lysis}-M) was similar between day and night (Table 2). On 8 September, B_{lysis}-L were 100 times and B_{lysis}-M was nearly 7 times higher during the daytime than in the nighttime incubations (Table 2). The percentage of bacteria lysed calculated from both methods was higher at day (Table 2).

Table 1. Non-diluted and diluted density of heterotrophic bacteria at the beginning of incubation at day and night. Values after ± are standard deviations of the three replicates of sampling.

Day	Field Heterotrophic Bacteria (10^5 cells mL^{-1})	Diluted Heterotrophic Bacteria (10^5 cells mL^{-1})
09/02	5.99 ± 0.39	1.31 ± 0.13
09/08	3.28 ± 0.068	0.79 ± 0.11
Night	Field Heterotrophic Bacteria (10^5 cells mL^{-1})	Diluted Heterotrophic Bacteria (10^5 cells mL^{-1})
09/02	4.78 ± 0.03	1.11 ± 0.04
09/08	3.40 ± 0.037	1.12 ± 0.19

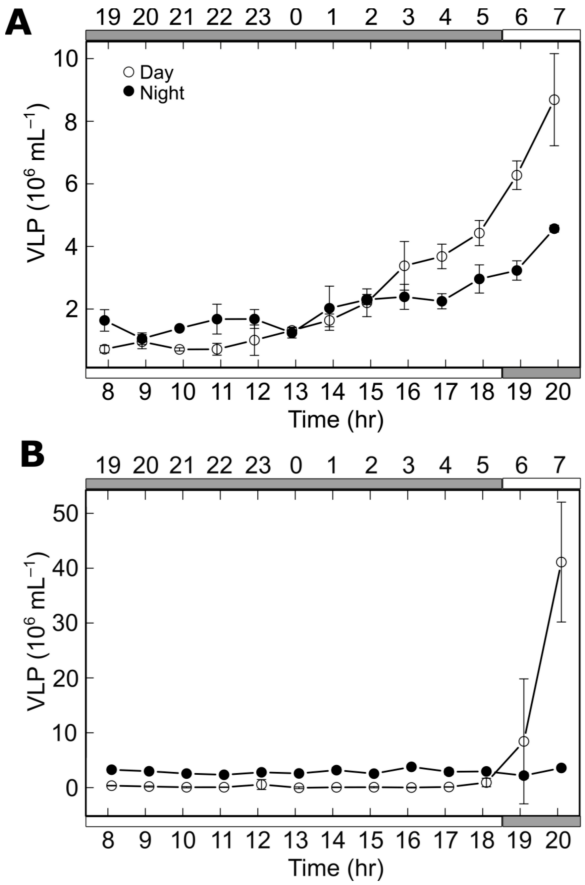

Figure 1. Temporal variation in density of virus-like particles (VLP). Daytime incubations from 8:00–20:00 (open circles and lower axis) and nighttime incubations from 19:00–7:00 the next morning (filled circles and higher axis). Experiments were conducted on 2 September 2020 (**A**) and on 8 September 2020 (**B**). Periods without sunlight are shaded. The standard deviation of VLP is labeled as a vertical line at each time point.

Table 2. Viral production (VP), bacterial mortality by viral lysis (B_{lysis}), and percentage of bacteria lysed (R_{lysis}). VP is calculated from the linear regression between VLP and time (VP-L) and arithmetic mean of VP during incubation (VP-M). B_{lysis}-L and R_{lysis}-L are calculated based on VP-L and B_{lysis}-M and R_{lysis}-M based on VP-M, respectively. The standard deviations of the three replicate incubations are presented after ±. Asterisk symbol indicates that the linear regression between mean VLP and incubation time is significant.

Day	VP-L (10^6 viruses mL^{-1} h^{-1})	VP-M (10^6 viruses mL^{-1} h^{-1})	B_{lysis}-L (10^5 bacteria mL^{-1} h^{-1})	B_{lysis}-M (10^5 bacteria mL^{-1} h^{-1})	R_{lysis}-L (% h^{-1})	R_{lysis}-M (% h^{-1})
09/02	2.61 * ± 0.41	2.37 ± 0.41	1.08 ± 0.17	0.99 ± 0.17	18.1 ± 2.2	16.5 ± 2.0
09/08	6.58 ± 0.84	14.64 ± 4.79	2.74 ± 0.35	6.10 ± 2.00	83.6 ± 9.0	186.0 ± 60.1
Night	VP-L (10^6 viruses mL^{-1} h^{-1})	VP-M (10^6 viruses mL^{-1} h^{-1})	B_{lysis}-L (10^5 bacteria mL^{-1} h^{-1})	B_{lysis}-M (10^5 bacteria mL^{-1} h^{-1})	R_{lysis}-L (% h^{-1})	R_{lysis}-M (% h^{-1})
09/02	0.92 * ± 0.11	1.96 ± 0.60	0.38 ± 0.05	0.82 ± 0.24	8.0 ± 1.0	17.1 ± 5.1
09/08	0.05 ± 0.02	2.28 ± 1.23	0.02 ± 0.007	0.95 ± 0.51	0.6 ± 0.2	27.9 ± 15.0

Our dilution incubations in two diel cycles indicated that the method chosen to calculate viral production depends on the linearity of the temporal increase in viral density. A linear increase in viral density is the fundamental assumption for calculating viral production by linear regression [15]. However, we found that the temporal increase in viral density during incubation was not always linear. Dilution incubation aims to cut the possibility of new viral infections [13,15], but if the diluted viral concentration is insufficiently low, viruses will infect host cells, and the density of free viruses will subsequently decrease. The new infections and release of viruses from newly infected cells cause the oscillations of viral density. In eutrophic, semi-enclosed coastal areas, high bacterial and viral density can lead to high contact rates and new infections and thus we need to be more cautious about the dilution ratio. In addition, the decreases in VLP during incubation can not only be caused by new infections but the decay of free viruses in seawater. To better assess the production of viruses, the decay of viruses during incubation should be considered [24]. Another caveat of this method is that bacterial host density was reduced by dilution, but the dissolved nutrient concentrations did not change. A lower bacterial density may mitigate the resource competition among bacteria and increase their metabolism and growth. The change of bacterial growth conditions would potentially alter viral infection and production. Unfortunately, we did not investigate the dynamics of the bacterial community during incubation to fully understand the effect of our dilution method on bacterial growth and viral production. This issue will be included in our future research on viral production. In conclusion, to apply the linear regression method of Winget et al. [15], the dilution ratio (20%) should be lowered to effectively stop new infections and host density and composition should be maintained, and thus keep the linearity of a temporal increase in viral density and the natural growth conditions of hosts.

Alternatively, the arithmetic mean of VP applies to estimating the viral production when viral density either oscillates with time or increases nonlinearly [22]. The VPs calculated by the two methods were most comparable when the VLP density increased linearly during the daytime incubation on 2 September. Although the linear regression was significant at nighttime incubation on 2 September, VP-M was twice as high as VP-L because the oscillation of viral density was stronger at night. On 8 September, the linear regression of VLP density with time severely underestimated the viral production at each period of increasing viral density in both daytime and nighttime incubations. Thus, calculating the VP at each period of increasing VLP is a better method to estimate VP as the temporal change in viral density is nonlinear [11,22].

The main cause of non-linearity of the temporal increase in viral density and exceptionally high VP-M during the daytime incubation on 8 September was the abrupt release of VLP in the evening (19:00–20:00). If we consider only VLP dynamics from 8:00 to 18:00, the daytime VP was not higher than nighttime VP (VP-L was 0.07 10^6 viruses mL^{-1} h^{-1}, R^2 = 0.038, p = 0.57; VP-M = 1.04 10^6 viruses mL^{-1} h^{-1}). On 8 September, the density of heterotrophic bacteria was lower (Table 1) and the small amount of VLP released would be difficult to detect during 8:00–18:00. Relatively lower VP before sunset and heterotrophic

bacteria density comparing with VP on 2 September would reflect the influence of tide: on 2 September, the coastal station was affected by spring tide and marine heterotrophic bacteria abundance increased and bacterial production could be stimulated, triggering higher lytic viral production [25]. The abrupt increase after sunset may be caused by specific cyanophages that adsorb to their host and replicate only at daylight and are released after six or eight hours of incubation [10]. Because of the larger burst size of cyanophages [26], their release in the late afternoon and evening would trigger a fast increase in viral production. Although we considered heterotrophic bacteria as the main host, the density of *Synechococcus* spp. was 1/10 of the density of heterotrophic bacteria at daytime in this semi-enclosed coastal station. Thus, we cannot exclude the possibility that the release of cyanophages contributed to viral production. The other possible explanation may be the strategy of viruses to reduce the harm caused by sunlight. To prevent the damage of viral DNA by UV light, viruses preferentially utilize DNA repair systems and reproduce within bacterial cells instead of bursting bacteria. Thus, less viruses are released during the daytime [27], and the release of viruses subsequently increases in the late afternoon or evening. However, the UV light intensity was similar on 2 and 8 September, and nutrient concentrations and microbial community composition were not recorded. Still, we cannot explain ecologically what triggered the abrupt increase in VLP in the evening that occurred only on 8 September.

From the two diel incubations, we found that VP was higher during the daytime than nighttime incubations on 2 September and on 8 September if we included the data during 19:00–20:00. One may argue that high VP during the daytime is due to high host density and growth rate, which enhances viral assemblage in host cells and increases the release of viruses [12,28]. However, in our study, bacterial density was not always higher at daytime (Table 1), and thus, the diel difference in viral production may not be explained by the diurnal rhythm of host density. This begs the question: what then causes the higher viral production during the daytime? Comparing with the bacterial mortality measured by Tsai et al. in this area (0.524–0.675 10^5 bacteria mL^{-1} h^{-1}, calculated from the viral lysis rate 0.047–0.300 h^{-1} in summer (July to September) and bacterial density of 5×10^5 mL^{-1} from [29]), the mortality calculated from mean burst size (24 viruses per cell) in this study is higher. This indicated that the real burst size in this area is higher than 24 and may also have diel variation. A possible explanation for the higher viral production may be the increase in burst size, which raised the release of VLPs per host cell [23]. It is known that burst size differs with the bacterial morphotypes [30]. Therefore, changes in bacterial community composition can be an important factor that influences the diel variation of burst size and viral production. Understanding the community composition of bacteria through sequencing and burst size by transmission electron microscopy will be necessary for the study of viral production in this subtropical coastal system.

Based on the possible diel patterns of viral production, we further provided insights into the diel change of nutrient release from microbial hosts through viral lysis and its potential influence on picophytoplankton diel dynamics and stoichiometry. We found that the mortality of hosts by viral lysis was higher at day than at night (Table 2, B_{lysis}-L and B_{lysis}-M). The percentage of bacteria lysed was also higher at day (Table 2, R_{lysis}-L and R_{lysis}-M on 8 September). With an understanding of the stoichiometry of bacteria [31], we can assess the nutrient recycled from lysed bacteria. Indeed, *Synechococcus* spp. density was found to increase during the day, reaching a summit in the late afternoon, and subsequently decreasing at night [32] in subtropical coastal seawater. The increase in *Synechococcus* spp. during the daytime is potentially supported by the high bacterial mortality by viral lysis. The release of P and N nutrients from lysis enhances the photosynthesis of *Synechococcus* spp. Shelford et al. [17] found that phytoplankton and cyanobacteria abundance is greater in incubations with in situ viral density than virus-diluted incubations, because of the release of ammonium from heterotrophic bacteria by viral lysis. One study by Tsai et al. [33] clearly showed that during the daytime, as much as 30% proliferation was observed for *Synechococcus* spp. in natural virus-containing samples, a proportion six times greater than that found in the virus-diluted

treatment groups (5%). Tsai et al. [33] also suggested that viruses exert significant effects on nutrient regeneration, enhancing daytime cell division rates in *Synechococcus* spp.

In summary, we investigated the diel variation in viral production and host mortality by viral lysis in a eutrophic marine system. We also examined two commonly used methods to calculate viral production. Viral production and viral lysis drive microbial dynamics, nutrient cycling, and biogeochemistry. We speculate that our research will improve the understanding and modeling of biogeochemical cycles and food web transfer efficiency in response to climate changes in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13090426/s1. The R code for viral production calculation and analysis: EstVP_Pub.r. Table S1: Data of virus-like particle (VLP) concentrations during the dilution incubations and bacterial density at the beginning of incubations on 2 September 2020. Table S2: Data of virus-like particle (VLP) concentrations during the dilution incubations and bacterial density at the beginning of incubations on 8 September 2020.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Dynamics of Virus-Like Particles in Three Replicates of Incubation

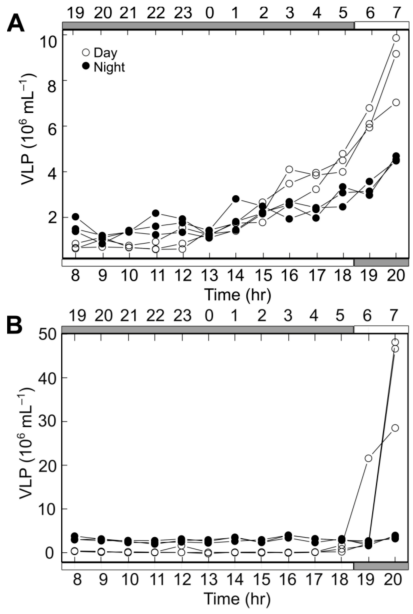

Figure A1. Temporal variation in density of virus-like particles (VLP) in three replicates of diluted incubation. Daytime incubations from 8:00–20:00 (open circles and lower axis) and nighttime incubations from 19:00–7:00 the next morning (filled circles and higher axis). Experiments were conducted on 2 September 2020 (**A**) and on 8 September 2020 (**B**).

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